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SECTION MEETINGS

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14070

Antithrombic Activity of Plasma: Quantitative Interrelationships.*

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In a previous publication[1] it was shown that the capacity of plasma to destroy thrombin depends fundamentally upon the heparin co-factor which is present. The role of heparin is essentially that of a catalyst. In the present paper we shall show that, within wide limits, the ability of plasma to destroy thrombin can be expressed by a relatively simple equation. The relationships thus established form a sound basis for the assay of antithrombin.

Materials and Methods. Seven parts of bovine blood, freshly drawn, were mixed with 1 part of 1.85% $K_2C_2O_4 \cdot 2H_2O$, and then centrifuged. Each cubic centimeter of the plasma contained 0.8 cc plasma plus 0.2 cc of the oxalate solution. Between experiments, the plasma was stored at –40°.

Heparin was purchased from Roche-Organon, Inc., Nutley, New Jersey. It was diluted with oxalated saline (0.85% NaCl + 0.09% $K_2C_2O_4 \cdot 2H_2O$) to make a concentration of 40 Toronto[2] units per cc.

The thrombin used in these experiments was prepared by methods previously

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* Aided by a grant from the John and Mary R. Markle Foundation. Funds were also supplied by the Graduate College, State University of Iowa.

[1] Seegers, W. H., *Science*, 1942, **96**, 300.

[2] Jaques, L. B., and Charles, A. F., *Quart. J. Pharm.*, 1941, **14**, 1.

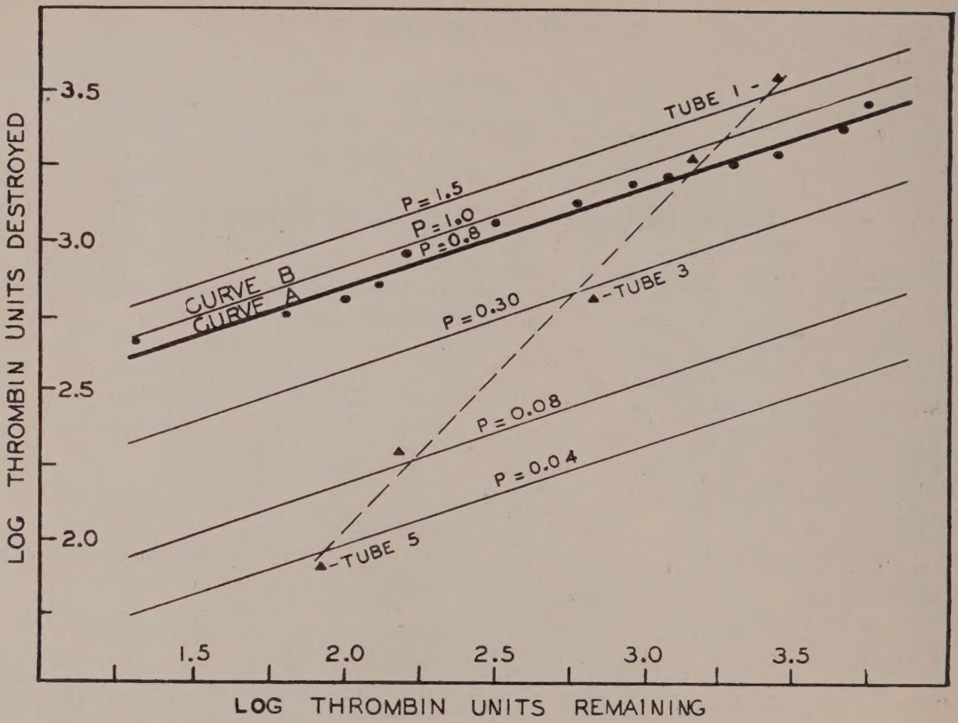

FIG. 1.

In each case, the initial thrombin, and also the mixtures of plasma and of thrombin, were assayed for thrombin titer as follows: A stock solution, A, is prepared by mixing 1 volume of isotonic imidazole buffer[5] with 2 volumes of 15% acacia (in 0.9% NaCl) + 6 volumes of saline (0.9% NaCl). Solution B is prepared by mixing 1.5 cc of solution A with 0.5 cc of the thrombin solution to be analyzed, the latter diluted suitably with oxalated saline (0.85% NaCl + 0.09% $K_2C_2O_4 \cdot 2H_2O$). To a 3 cc tube, add 0.1 cc fibrinogen solution[4] + 0.4 cc of solution B. The clotting time is noted. By variable dilution and by interpolation one determines to what extent the thrombin solution must be diluted, in preparing solution B, in order that the final clotting mixture will coagulate in exactly 15 seconds. The product of all the various dilutions involved in the titration, multiplied by the correction factor, 1.5 (see text), represents the number of thrombin units in the specimen being analyzed.

described;[3] it was free of antithrombin, and it had a potency of 8000 units per mg N. It was dissolved in oxalated saline (0.85% NaCl + 0.09% $K_2C_2O_4 \cdot 2H_2O$).

In making the assay of thrombin, the thrombin unit is defined[4] as the amount of activity required to clot 1 cc of standard fibrinogen solution in 15 seconds. This standard fibrinogen solution contains buffer, a supplement of colloid, and an optimum amount of calcium chloride. In the present experiments, however, it was necessary to have oxalate present in order to prevent conversion of prothrombin into thrombin. Under these conditions, thrombin reacts more sluggishly with fibrinogen than when free calcium is present.[4] It has been found possible to correct this deviation from standard units by the use of a multiplication correction factor, 1.5. The results recorded in the present paper have been subjected to this correction, and the units are therefore the same as those previously used in this laboratory.

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[3] Seegers, W. H., *J. Biol. Chem.*, 1940, **136**, 103.

[4] Seegers, W. H., and Smith, H. P., *Am. J. Physiol.*, 1942, **137**, 348.

[5] Mertz, E. T., and Owen, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 204.

Results. To each of a series of tubes were added 1 cc of oxalated plasma, 0.1 cc of heparin solution, and variable amounts of thrombin. Oxalated saline was added in amounts sufficient to give a total volume of 4.0 cc. Under these conditions, the total amount of thrombin eventually destroyed is a function of the amounts of thrombin and of plasma (co-factor) used. The heparin added merely accelerates the reaction.[1] In the present experiments, heparin was present in sufficient amounts to complete the reaction within a very few minutes; nevertheless, an entire hour was allowed before the remaining thrombin was measured. During that time the fibrin was allowed to remain in the mixture.

In plotting thrombin destruction (Fig. 1) it was discovered that a linear relationship exists (Curve A) when the logarithm of thrombin destruction (Y) is plotted against the logarithm of thrombin remaining (X). The equation expressing this relationship may be written as follows:

$$\log Y = K + n \log X \tag{1}$$

This equation also applies if heparin is not added to the reaction mixture. Under those circumstances much more time is required before equilibrium is reached.

In the above experiment, the individual tubes contained variable initial amounts of thrombin, but constant amounts of plasma. In each case the plasma (1.0 cc) contained oxalate solution; in reality each tube contained only 0.8 cc of plasma. A number of experiments were then made, using several fixed amounts of thrombin, but variable amounts of plasma with each. At each thrombin level tested, thrombin destruction was always more pronounced when larger amounts of plasma were added. Empirically it was determined that the equation, already given, could be modified to include variable amounts of plasma (P):

$$\log \frac{Y}{P} = K + n \log \frac{X}{P} \tag{2}$$

In using the data already given in Curve A, Fig. 1, the constants for this equation can be calculated. The equation can then be written in the following form:

$$\log \frac{Y}{P} = 2.235 + 0.336 \log \frac{X}{P} \tag{3}$$

With the aid of this equation, we have plotted, in Fig. 1, a series of parallel lines representing theoretical values with different amounts of plasma. The values plotted represent plasma volumes varying from 0.04 cc to 1.5 cc.

In addition to the experimental results centering about curve A, Fig. 1 also shows one group of our experimental points, obtained with variable amounts of plasma. Thus, tube 1 contained 1.52 cc; No. 3, 0.32 cc; No. 4, 0.08 cc; No. 5, 0.04 cc. It will be seen that the experimental points lie quite close to the theoretical thrombin levels indicated by the parallel lines which had been constructed on the basis of equation 3.

It might be added that the constants in equation 3 are somewhat different when highly impure thrombin is used. Evidently, certain impurities affect the reaction to some extent.

Discussion. The equation, expressing the logarithmic relationship between thrombin and co-factor, is at present entirely empirical. A preliminary attempt by Doctor George Glockler of the Department of Chemistry to reconcile the equation with traditional reaction mechanics has given encouraging results, which are, nevertheless, still incomplete.

The results which we have presented provide a sound basis for assay of antithrombin (*i.e.*, of the co-factor). By determining the values of X and of Y one can readily determine the value of P. From the standpoint of precision, the relative amounts of thrombin and of plasma which one mixes should be so adjusted that approximately 50% of the thrombin will be destroyed. From the equation, or from the chart, the co-factor titer is readily calculated, and expressed in percentage of the normal plasma, upon which the equation and the chart are based. If, instead of these relative values for the co-factor, one wishes to express results as units, one can select a unit standard based either upon a definite amount of plasma, or upon definite percentage destruction of a fixed amount of thrombin.

Summary. In the destruction of thrombin by plasma "antithrombin," heparin serves merely as a catalyst. The amount of thrombin eventually destroyed by a given amount of plasma is dependent fundamentally upon the amount of co-factor present. The concentrations may be expressed by the following equation:

$$\log \frac{Y}{P} = 2.235 + 0.336 \log \frac{X}{P}$$

where Y is the amount of thrombin destroyed, X is the amount of thrombin remaining, and P is the amount of plasma (bovine) present in the reaction mixture.

14071

Reduction of Blood Pressure of Hypertensive Rats by Administration of Certain Marine Oils.*

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Our studies on the nature of the substance present in renal extracts which is effective in reducing the blood pressure in experimental hypertension have demonstrated that this active principle is of relatively small molecular weight.[1] This is evident from its chemical and physical properties as well as its effectiveness when administered orally. It was hoped, therefore, that sources other than mammalian kidney, in which the active principle is present in only small amounts, might be found as a more practical source of the material. Attempts to obtain active extracts from the kidney of fishes and amphibia have up to the present been unsuccessful. However, we have not been able to obtain large amounts of fresh tissue from these sources. Since these experiments have been in progress, there has appeared a recent report of Friedman, Soloway, Marrus and Oppenheimer,[2] in which certain quinones were demonstrated to have the capacity to reduce the blood pressure. We have been able to confirm these results. There has also appeared the report of the effectiveness of vitamin A,[3] which we have found to be relatively inert. It suggested itself, therefore, that the active material might be a derivative of the vitamin itself or one of the contaminants present in fish oils from which vitamin concentrates are prepared.

Methods. Rats have been used throughout in the present study. They were rendered hypertensive by fixation of the kidney by looping a stout cotton thread over the poles in the shape of a figure 8.[†] The blood pressures were determined by the plethysmographic method.[4]

The materials used in the present study consisted of pure provitamin A in the form of a mixture consisting of 90% β-carotene and 10% α-carotene and preparations and concentrates of various fish oils. These were administered orally by admixture with the animal's food. In addition to the preparations used in their natural form, some of the preparations were subjected to the following procedures which resulted in the destruction of vitamin A.

(1) Aeration by means of a current of air

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* Aided by grants from the John and Mary R. Markle Foundation, from the National Life and Accident Insurance Company and from Mr. Joe Werthan.

[1] Grollman, A., Harrison, T. R., and Williams, J. R., *Fed. Proc.*, 1934, **1**, 34; in press.

[2] Friedman, B., Soloway, S., Marrus, J., and Oppenheimer, B. S., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 195.

[3] Pena, J. G., and Villaverde, M., *Rev. Cubana Cardiol.*, 1940, **2**, 322.

[†] This simplified procedure has proved to be superior to that in which silk is applied to the kidney, as originally suggested by Page.

[4] Williams, J. R., Grollman, A., and Harrison, T. R., *J. Clin. Invest.*, 1939, **18**, 373.

TABLE I.

Effect of Various Vitamin A Preparations and Marine Oils on Blood Pressure of Hypertensive Rats. Each experiment was performed on a group of 5 animals; the values in table refer to amounts received by each animal and average drop in blood pressure observed.

				Systolic blood pressure in mm mercury		
					Reduction during week following beginning of treatment	
Substances tested	Procedure to which test material was subjected	Quantity admin., g per rat	Original vit. A content U.S.P. units × 1000	Pre-treatment level	Daily avg*	Max.†
β-carotene	—	0.2	200	180	0	2
Vit.-A concentrate	—	1.6	500	172	8	17
” ”	Oxidation with H_2O_2	1.6	500	168	6	10
” ” (alcohol)	—	0.4	400	191	5	10
” ” ”	Oxidation with CrO_3	0.4	400	170	4	8
” ” (ester)	—	0.4	400	174	0	0
Cod liver oil	—	12.0	40	170	0	2
” ” ”	Aerated at 100°	12.0	40	162	0	3
” ” ”	Ultraviolet irradiation	12.0	40	169	0	5
” ” ”	Oxidation with CrO_3	6.0	20	172	12	14
” ” ”	” ” ”	5.0	8	186	16	24
” ” ”	” ” H_2O_2	12.0	40	178	15	18
Crude fish oil	—	16.0	low	170	0	2
” ” ”	Oxidation with CrO_3	16.0	”	182	15	22
Sardine body oil (residue)	—	8.0	”	155	10	19
Sardine body oil (glyceride fraction)	—	8.0	”	168	0	4
Sardine body oil (fraction high in vit. A)	—	8.0	high	172	13	20
Sardine body oil (fraction low in vit. A)	—	8.0	low	180	12	20

*The values in this column were obtained by adding the differences between the pretreatment level and the observed levels on each day and dividing by the number of observations.

†The values represent the maximum average drop per rat observed on any day during the course of therapy.

for 10 hours, during which time the preparation was kept in a water bath at 100°C. (2) Irradiation with ultraviolet light from a mercury arc quartz lamp for one hour at a distance of 50 cm. (3) Oxidation with hydrogen peroxide (3% and 30%) in alkaline solution. The marine oil was stirred rapidly for 2 hours with an equal volume of 3% aqueous hydrogen peroxide, or with 10 cc of a 30% hydrogen peroxide solution with the addition of a drop of concentrated aqueous sodium hydroxide. The resulting emulsion was mixed with the animal's food and fed after drying in a current of air at room temperature. (4) Oxidation with CrO_3 in glacial acetic acid solution. To 20 cc of the marine oil was added 1 g of CrO_3 dissolved in the least possible amount of distilled water and 20 cc of glacial acetic acid. The mixture was stirred rapidly for 2 hours; made slightly alkaline with aqueous sodium hydroxide. After adding 100 cc of distilled water, the mixture was extracted repeatedly with ethyl ether and the resulting solution, after washing twice with a little water, was distilled *in vacuo* at 30°C. The remaining oil was mixed with the animal's food and administered in this form.

In addition to carotene, the following preparations have been studied: a concentrate of vitamin A in natural fish liver oil, containing 200,000 units per cc; a sample of esterified vitamin A, containing 500,000 units per g; and a sample of the free alcohol, containing a million units per g; codliver oil containing 1600 and 5000 units per g; a fish liver oil containing 60,000 units per g; ordinary commercial fish oil, dogfish oil, and sardine body oil. The last two named substances were tested in their original form and in fractions high in vitamin A, low in vitamin A, glyceride fraction, and residues,[‡] as prepared commercially by distillation.

The substances tested were administered orally in all cases by mixing them with the animal's usual food. The mixture thus obtained was readily consumed by the animals without wastage.

Results. In the accompanying table are reproduced such of the results as bear on the present problem. It is evident from these results that a variety of preparations derived from fish body or liver oils is capable of reducing the blood pressure of hypertensive rats. The results, however, indicate that the activity of different preparations is not proportional to their vitamin A content. In fact, oxidation which completely destroys all vitamin A activity enhances the effectiveness of the preparations in reducing the blood pressure. Destruction of vitamin A by heat and atmospheric oxygen does not increase this activity but more intensive oxidation by H_2O_2 or CrO_3 does so. Oxidation of vitamin A concentrates increases the activity little or not at all, the degree of activation depending rather upon the volume of oil used and its source, and not upon its original vitamin A content.

It would appear from the results of Table I that the agent responsible for the observed reduction in blood pressure is not derived from vitamin A but resides in some other constituent of fish oils which happens to be concentrated in commercial preparations of vitamin A. This would account for the observations of Pena and Villaverde[3] and of Wakerlin, Moss and Smith,[5] who have noted the capacity of vitamin A concentrates to reduce the blood pressure of patients and hypertensive dogs.

Refined fish oils, particularly those of marine origin, are more prone to auto-oxidation than the crude oils from which they are derived.[6] This would account for the presence of the material effective in reducing the blood pressure in the highly refined vitamin A concentrates, whereas, they are found in cruder preparations only after oxidation. On the other hand, the most highly purified vitamin A concentrates, in which there is only a small amount of residual fish oil, are relatively inert.

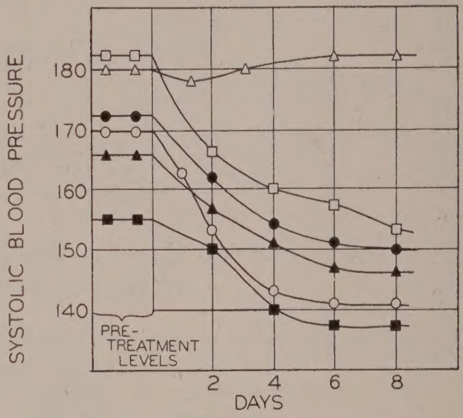

FIG. 1.

The effect of renal extract and various marine oils on the blood pressure of hypertensive rats.

Ordinates represent the systolic blood pressure in millimeters of mercury; abscissæ, time in days following a period of observation prior to starting treatment. All values represent averages obtained on groups of 5 rats, each series of which received the following admixed with their food on days 0 and 2 of the experimental period:

○ 20 g of renal extract derived from 50 kg pig's kidney.

● 22 g of cod liver oil containing 20,000 units of vitamin A after oxidation with CrO_3.

□ 40 g of crude fish oil oxidized with CrO_3

■ 40 g of a residual fraction of sardine body oil.

△ 40 g of cod liver oil containing 200,000 units of vitamin A after irradiation with ultraviolet light.

▲ 40 g of a vitamin A rich fraction of dogfish liver oil.

To conserve space the protracted return of the blood pressures to their pre-treatment levels is not indicated on the chart.

Discussion. Until the respective substances are isolated, it is impossible to conclude about the relationship between the antipressor substances present in fish oils and kidney extracts. Preliminary studies demonstrate many similarities between the two as regards their general physical and chemical properties. They are almost identical insofar as their effect on the blood pressure is concerned, as shown in Fig. 1, which reproduces typical experiments not shown in Table I. Both are effective orally, manifest their action slowly and exert a relatively prolonged effect (a week or more) compared to the evanescent action which follows the depressor action of other substances.

[‡] We are indebted to Mr. A. Lee Caldwell of the Eli Lilly and Company for the cod liver oil and vitamin A (alcohol and ester) concentrates; to Dr. L. M. Fox of the Winthrop Chemical Company for vitamin A in fish oil concentrate; to Dr. K. C. D. Hickman and the Distillation Products, Inc., for the dogfish and sardine oil fractions; and to Mr. W. V. B. Potter and the Beaufort Fisheries, Inc., for the crude fish oil.

[5] Wakerlin, G. E., Moss, W. G., and Smith, E. L., *Science*, 1942, **96**, 161.

[6] Buxton, L. O., *Ind. and Eng. Chem.*, 1942, **34**, 1486.

The relatively high potency and ready availability of crude fish oils render these more satisfactory than kidneys as a source of the active principle effective in hypertension. Further experiments on the nature of the active substance are in progress. It is possible that a quinone analogous to those found to be effective by Friedman *et al.*[2] is formed by the oxidation of marine oils and that this is the agent responsible for the observed reduction in blood pressure.

Summary. Fish body and liver oils contain a substance which is effective in reducing the blood pressure of hypertensive rats. The effectiveness of these oils is increased by oxidative procedures which destroy vitamin A. The observed activity appears to be associated with some substance, other than vitamin A, present in fish oils. Fish oils offer greater promise than kidneys as a possible source of a therapeutic agent effective in reducing the blood pressure in hypertension.

14072

Precipitation and Concentration of Influenza Virus With Alum.*

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Influenza vaccines in which the virus has been concentrated by various methods produce a better antibody response than unconcentrated preparations.[1,2] Concentration of virus from the allantoic fluid of infected chick embryos has been accomplished by lyophilizing,[1] by adsorption on precipitates which result from thawing frozen extra-embryonic fluids,[2,3] by adsorption on and elution from chicken erythrocytes,[4] and, with mouse lung preparations, by adsorption on a precipitate of calcium phosphate and solution in sodium citrate.[5]

Alum has been used in the preparation of vaccines from bacteria and bacterial toxoids especially to increase the immunizing efficiency. This paper will describe a method for alum precipitation of the virus of influenza grown in the allantoic fluid of chick embryos.

Preparation of Virus Suspensions. Virus suspension diluted to 10^{-4} was inoculated into the allantoic cavity of 12-day-old chick embryos. The embryos were then incubated at 37°C for 2 days, candled, and the live eggs placed at 4°C overnight. Chilling the embryos facilitated collection of the allantoic fluid, and admixture of blood cells was avoided. The strain PR8 of type A influenza virus and the strain Lee of type B virus were used.

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† Associate Bacteriologist, United States Public Health Service, assigned to California State Department of Public Health.

[1] Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1942, **75**, 495.

[2] Hirst, G. K., Rickard, E. R., and Whitman, L., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 129.

[3] Hare, R., McClelland, L., and Morgan, J., *Canad. Pub. Health J.*, 1942, **33**, 325.

[4] Francis, T., Jr., and Salk, J. E., *Science*, 1942, **96**, 499.

[5] Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 709.

TABLE I.
Alum Precipitation of Influenza Virus in Allantoic Fluid of Chick Embryos.

Strain used	Vol. of fluid, cc	Amt of 10% $NaHCO_3$ added, cc	Amt 10% alum added, cc	pH	Titer of supernatant	Titer of ppt. conc. 10X
PR8	15	—	.3	6.7	160	1280
Titer before	15	.05	.4	6.7	80	3800
treatment = 640	15	.10	.5	6.7	40	5120
Lee	10	—	.2	6.7	480	5
Titer before	10	—	.3	6.3	320	320
treatment = 480	10	—	.4	6.0	160	1280
	10	.10	.5	6.3	40	2560

Titration of the Virus. Quantitative estimations of the virus content of allantoic fluid and of the redissolved alum precipitates were made by a modification of the chicken cell agglutination technic.[6]

Precipitation of Influenza Virus with Alum. Infected allantoic fluid was treated with varying amounts of a 10% solution of $Al_2 (SO_4)_3 K_2 SO_4 \cdot 12H_2O$ in order to determine the least amount needed to produce maximum precipitation. The precipitate, which formed within a few seconds after the alum was added, was centrifuged at about 3,000 r.p.m. for 20 minutes in the horizontal head of the centrifuge. The supernatant was decanted and the precipitate washed 3 times in sterile saline. Tests on the first washing revealed only insignificant amounts of virus probably contained in the fluid adhering to the precipitate. Second and third washings did not agglutinate chicken cells in dilutions greater than 1:10 and many contained no demonstrable virus. The washed precipitates were resuspended in a volume of saline equal to 1/10 the volume of the original extra-embryonic fluids. Merthiolate at a dilution of 1:10,000 was used as a preservative.

The alum-precipitated virus readily dissolved in 20% sodium citrate and the resulting solution, restored to the original volume in saline, agglutinated chicken erythrocytes to about the same titer as the original fluids. The mouse infectivity of the redissolved precipitates was only slightly diminished.

The results of experiments on precipitation of strains PR8 and Lee are shown in Table I. Strain PR8 was precipitated with less alum than was required for strain Lee and generally a smaller loss of virus occurred with PR8 than with Lee. Of 14 attempts to concentrate PR8 tenfold, yields of 80% or over were obtained in 10 experiments. With strain Lee, however, an 80% yield was obtained in only 2 out of 8 preliminary experiments. The unsatisfactory yields, particularly with strain Lee, can be explained, in part at least, by the acidity which developed during the precipitation. pH measurements were made with indicators. At pH below 5.0 the antigenicity of influenza virus is destroyed.[7] When .5 cc of alum was added to 15 cc of fluid the pH reached 6.0 or below, and unless the acid was neutralized the agglutination titer was reduced. The addition of sodium bicarbonate brought the pH near to 6.5 with little effect on the amount of alum required to precipitate the virus.

Effect of Inactivation with Formaldehyde. Inactivation of the virus with formaldehyde had little or no effect on the precipitation of virus with alum. The allantoic fluids were treated with 0.25% formaldehyde at a temperature of 5°C for 24 hours. In some instances the formaldehyde slightly reduced the titer of the fluids. The formalinized virus was then tested for infectivity in mice, precipitated, washed, and resuspended as previously described.

Immunization of Mice. Sets of 8 mice were inoculated intraperitoneally with 0.5 cc of graded five- or tenfold dilutions of the material to be tested. For controls, mice were inoculated with 0.5 cc of 10% horse serum broth.

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[6] Bodily, H. L., and Eaton, M. D., *J. Immunol.*, 1942, **45**, 193.

[7] Eaton, M. D., *J. Immunol.*, 1940, **39**, 43.

TABLE II.
Results of Immunization of Mice with Alum Precipitated Influenza Virus.

		Lesions in mice immunized with dilutions of*				
Strain	Preparation used	1:10	1:50	1:250	1:1250	1:6250
PR8	Untreated allantoic fluid	7.5	0	15	27.5	47.5
”	Formalinized and precipitated with alum	—	15	25	95	100
Lee	Untreated allantoic fluid	0	0	0	6.6	25
”	Formalinized and precipitated with alum	0	2.5	0	25	33

*Results given as percentage of lung tissue showing consolidation in each group of mice. Controls: 90-100% consolidation.

Two weeks later all the mice were inoculated intranasally with 0.05 cc of a dilution of mouse-adapted virus containing from 10 to 100 m.l.d. Deaths were recorded during a 10-day period at which time the remaining mice were killed and the extent of lesions in the lungs recorded. The results of some of the immunization experiments are given in Table II. The inactivated alum-precipitated suspension concentrated 10 times appeared to be slightly less antigenic in mice than the untreated virus. Most of this reduction in the antigenicity was due to the fact that inactivation with formaldehyde impaired the immunizing power of the unconcentrated fluid.[7]

Stability of the Alum-precipitated Virus. Alum-precipitated suspensions were stored at 4°C and –70°C and tested at intervals during a period of 3 months. At both temperatures the suspensions when redissolved in sodium citrate retained practically the same agglutination titer as they possessed when prepared. One sample of alum precipitated virus retested in mice after an interval of about 3 months was found to have retained its immunizing power. Some of the preparations lost part of their agglutinative activity and pH measurements on these indicated that acidity was responsible for the reduction in titer.

Concentration of Virus with Chicken Cells. Recently Hirst[8] has observed that influenza virus can be eluted from agglutinated chicken cells. This observation has been applied by Francis and Salk[4] to the concentration of influenza virus. It was of interest to know if influenza virus concentrated in this manner could be precipitated with alum.

Allantoic fluid infected with strain PR8 was treated with sterile chicken cells to a final concentration of 1% and placed at 4°C for one hour. The suspension was then centrifuged at low speed, the cells resuspended in ⅕ of the original volume of allantoic fluid, and incubated at 37°C for 2 hours with intermittent shaking. At the end of the incubation period the suspension was again centrifuged and the supernatant carefully removed. By this method the virus was concentrated almost quantitatively.

Fluids infected with strain Lee gave essentially the same results. In this case, however, 2.5 to 3.0% cells were required to remove the virus from the fluid instead of 1% required by strain PR8.

Following concentration with chicken cells the fluids were treated with formaldehyde and precipitated with alum with no more difficulty than was experienced on untreated fluids. The few attempts to produce alum-precipitated virus by this method have given better yields than were obtained with alum precipitation alone.

Immunization of Human Beings. Six human volunteers were inoculated subcutaneously with 1.0 cc of a mixture of equal parts of alum precipitates of PR8 and Lee strains concentrated tenfold by the combined method. A comparable group was inoculated with type A and B vaccine received from Dr. Hirst and prepared by adsorption to the precipitate of allantoic fluid.[2] Blood specimens were collected before and 2 weeks after vaccination, and the antibody titers determined by the agglutination-inhibition technic with chicken erythrocytes. The results of these preliminary tests as presented in Table III indicate that the alum precipitates produce a very definite antibody response.

Discussion. The possible value of alum-precipitated influenza virus as a vaccine is suggested by these experiments. By this

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[8] Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

TABLE III.
Antibody Response of Persons Receiving Concentrated Influenza Vaccines.

Vaccine	No. tested	Mean titers type A		Mean titers type B	
		before	after	before	after
Alum ppt.	6	28.5	406	16.0	362
Hirst method[2]	8	29.3	430	32.0	234

method the virus is separated from the formaldehyde used in inactivation, and from other extraneous materials. The continued action of formaldehyde apparently tends to diminish the antigenic potency of influenza vaccines. Formalin also produces some discomfort after subcutaneous inoculation. Suspensions of the alum precipitates are relatively stable at 4°C without the necessity of drying. By the combined method, using adsorption and elution from chicken cells and precipitation with alum, vaccines more concentrated than those now in use could be made.

Summary. The virus of influenza grown in allantoic fluid of chick embryos can be precipitated in a stable form with alum and the precipitate redissolved in sodium citrate. This procedure does not alter the infective, antigenic, or hemagglutinating properties of the virus. Adsorption on sterile chicken cells was also used to concentrate the virus before inactivation and precipitation with alum.

The immunizing power of formalinized and alum-precipitated virus in mice was slightly less than that of active fluid. Inactive concentrated alum precipitates inoculated subcutaneously produced a satisfactory antibody response in human beings. The suspensions of alum precipitates freed of formaldehyde and acid retain their antigenicity when stored at 4°C for at least three months.

14073

Method of Producing Open Skin Wounds Uniformly Infected with Staphylococci, Suitable for Local Chemotherapy Studies.*

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While engaged in the evaluation of various methods of local sulfonamide chemotherapy of infected wounds, the problem arose of producing controlled, equally involved, infected skin wounds, before different methods of local chemotherapy could be compared.

In evaluating the effectiveness of local chemotherapy of infected wounds, quantitative bacteriological studies were first considered. At first thought, it might be believed that the wounds could be excised completely, ground up in a tissue mill, and the degree of infection estimated by bacterial population of the macerates. This method is open to the criticism that in excising the wound, some normal tissue necessarily must be included, making it impossible to obtain quantitatively reproducible results. Moreover, it is extremely difficult to grind the skin of animals either by sterile mechanical mills, or by freezing-grinding methods. It was finally decided that for all practical purposes, epithelization rate, healing time, and qualitative bacteriological studies would be the best ways in which to evaluate the effectiveness of local chemotherapy of infected open wounds.

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* Supported in part by a grant from the Graduate School of the University of Minnesota, and by the General Research Fund of the University of Minnesota.

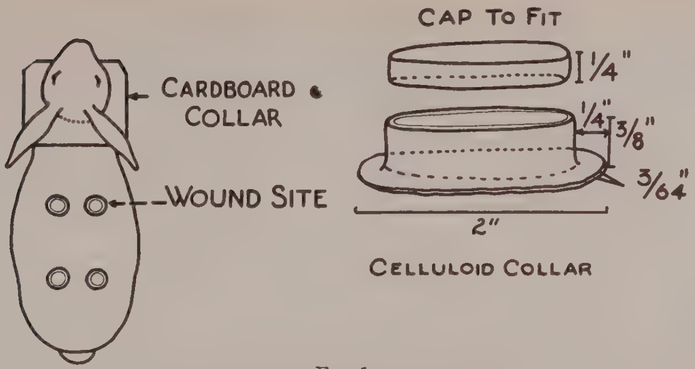

FIG. 1.
Wound dressings and restraining collars.

Previously[1] one of us has briefly described a technic which was developed for studying the rate of granulation and epithelization of clean, open skin wounds in rabbits. The present method is essentially an extension of this method to infected wounds.

Methods. Three to 5-lb healthy rabbits were anesthetized by intravenous sodium pentobarbitol, using picrotoxin if necessary when barbiturate poisoning seemed imminent. The fur along the back and sides was clipped close to the skin with electric hair clippers. The best type found was the Oster No. 2 small animal clippers, with "Angra" (angora) head and blades.[†] Special celluloid collars open at the top and bottom, and flanged at the bottom, and with separate celluloid caps to fit, were heat moulded. They are illustrated in Fig. 1. The flanges of the collars were cemented to rings of single layers of cotton gauze, with flexible U.S.P. collodion, and allowed to dry. The flanges of the collars and the gauze rings were cemented to the clipped skin of the animals with flexible collodion, the collars being held firmly to the skin with weights until thoroughly dry. Electric hair driers or electric lamps hastened the drying. Such protective dressings could be covered with the removable celluloid caps, which then could be removed easily for inoculation, examination and measurement of the wounds, change of dressings, and taking of smears. The collars remained firmly cemented to the skin for 2 to 3 weeks if properly applied. In rabbits, hair growth was not noticed under the flanges, but it did occur in guinea pigs and subsequently caused the collars to be lifted away from the skin. Hair growth did occur inside the collar around the wound. Cardboard collars, as illustrated in Fig. 1, were placed around the rabbits' necks to prevent tearing at the dressings. These collars were held in place on the animal with adhesive tape, and had to be watched carefully, as some rabbits eventually could remove them. In most cases they did not prevent the animals from feeding and drinking. The celluloid wound dressings demanded frequent inspection, as leaks sometimes developed, especially if the animal freed itself of the cardboard collar and gained access to the wounds. In the event of leaks, patches of gauze and collodion were applied.

The operating room atmosphere was treated with propylene glycol vapors during the operations. The skin area inside each collar was then treated with Novak's germicide solution,[‡] and circular skin wounds were made with sterile instruments, cutting down to the panniculus carnosus muscle layer, exercising care to avoid blood vessels. These wounds were approximately 3 to 4 mm in diameter. Small curved scissors and tissue forceps were found

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[1] Olson, M., Slider, E., Clark, W. G., and Macdonald, R., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 396.

[†] John Oster Mfg. Co., Racine, Wisconsin.

[‡] 95% alcohol 515 g, acetone 100, water 375, saponated cresol 10, mercuric chloride 0.7, Eosin Y 0.6, acid fuchsin 0.08.

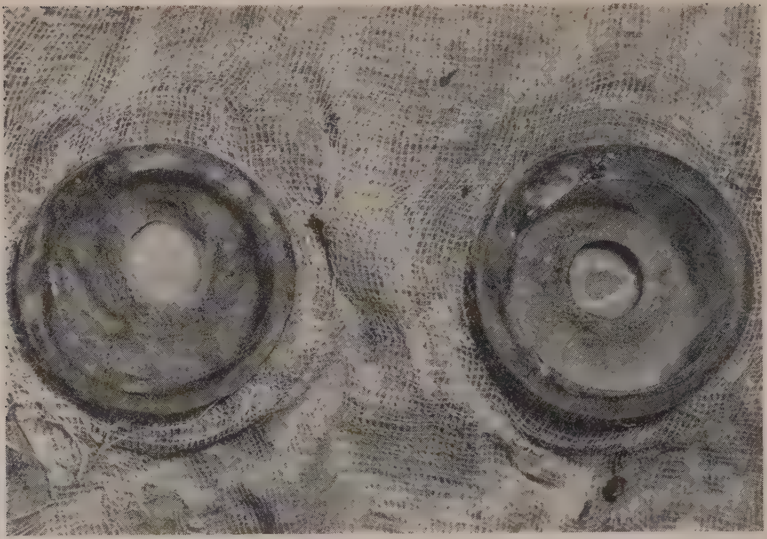

FIG. 2A.
Photograph of 2 wounds, 24 hours after inoculation with staphylococci.

to be the best instruments for producing wounds of uniform size in the collars, although several types of rotating cylindrical cutting tools were constructed and tried but were unsatisfactory due to the mobility of rabbits' skin. Usually within 6 days such wounds, if kept moist, stretched to approximately 1 cm or more in diameter, after which they began to epithelize and close. In the rabbit, clean skin wounds always close much more rapidly when they are dry, but it was necessary to keep them wet for these first studies, hence the method was standardized for moist wounds. After the stretching period, all the wounds were usually uniform in size, as illustrated in Fig. 2A, but practice and care were necessary before uniform wounds could be reproduced. Four such wounds usually were produced on each rabbit, in the skin over the large back muscle bundles of the multifidus and sacrospinalis, 2 wounds on each side of the animal, as close to the mid-dorsal line as possible without overlapping the dressings on the other side of the animal.

An inoculum of 10 cc of a 24-hour culture of *Staphylococcus aureus* in peptone water was poured into 1000 cc prescription bottles containing 100 cc of sterile nutrient agar which had been allowed to solidify on the broad flat surface of the bottle, thus affording a maximum of surface for bacterial growth. After the solution was uniformly spread over the agar, the bottle was incubated for 24 hours at 37°C. At this time 50 cc of sterile saline was used to wash off the growing organisms. To this wash fluid, 50 cc of a sterile 4% aqueous "methocel" gel was added. The methocel was a pharmaceutical grade of synthetic methyl cellulose ether,§ essentially inert, non-toxic, neutral, and of a specific gravity of 4000 centipoises for a 2% solution at 20°C. After thorough stirring, this suspension of the organisms in the gel was used for inoculation. Approximately 10 cc of the suspension was introduced into each celluloid collar and on the freshly made wound, and the celluloid caps replaced firmly. Each inoculum contained approximately 20 billion organisms as determined by duplicate plate counts.

After such an inoculation, the wounds were not touched for about 4 days, at which time they were uniformly infected. The infections were allowed to develop, and then treated with various forms of therapy, two serving as experimental and 2 as control wounds. The therapeutic agents were dissolved in and made up to 2% sterile methocel. The gels were changed every other day and the dimensions of the wounds measured at this time with sterile protractors. Both the X and Y axes were measured, if the wounds were not perfect circles, and the areas usually calculated. Treatment was continued until the wounds healed so that the time to healing by secondary intention could be determined.

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§ The Dow Chemical Co., Midland, Mich.

Ointments of various kinds, and other types of gels such as gum karaya or tragacanth, whether glycerated or not, were not as satisfactory as methocel for inoculation or treatment because many of them cause skin maceration. Maceration due to the aqueous gel being confined under fairly air tight conditions was not a major consideration. All types of methocel were not satisfactory because of more or less maceration. Pure pharmaceutical grades should be used.

The problem of attempting to devise a method of standardly infecting wounds was twofold, first to secure a localized ulcer which would not spontaneously regress, and second to fix the organisms in the skin lesion so that they would not invade the blood stream to produce a fatal bacteremia before termination of the chemotherapy studies. Occasional strains of staphylococci isolated from the human skin ulcers, when inoculated directly into rabbit skin wounds, satisfied the first condition described above, but fatal bacteremia frequently resulted, making these strains unsuitable for our purpose. At first, attempts were made to increase the virulence of the organisms for the type of wound described, by repeated intravenous passage through rabbits. This gave little improvement over the virulence of strains directly inoculated after isolation from human sources. Then various combinations of mucin, methocel, and staphylococci as the inocula were tried, because of the striking action of mucin in enhancing the virulence of various bacteria.[2] Mucin‖ did not seem to satisfy the first and second requisites described above. It has been long known that filtrates of staphylococcus cultures may contain a dermotoxin which produces skin ulcers.[3] Therefore it was thought that repeated passage through skin might increase the affinity of the organisms for that tissue so that they would produce localized, ulcerating lesions of the type desired. This proved to be the case.

Several strains of hemolytic, coagulase-positive staphylococci were obtained from various types of clinical skin infections in the University Hospital,¶ and one from Doctor T. F. Nicholson, of the University of Toronto.** The latter strain had been used for studies of skin infection in rabbits.[4] The organisms were inoculated intradermally into shaved areas on the backs of 6- to 8-week-old rabbits, and after 24 to 48 hours, the organisms were recovered from the resulting abscess, or if no abscess developed, from the general area inoculated. They were then isolated in pure culture and kept on blood agar for no longer than 2 weeks, before wounds were inoculated with the staphylococcic suspensions in methocel, as previously described. Thereafter the virulence and skin affinity of the strain was maintained merely by isolation of the organisms from the open experimentally infected wounds every two weeks.

It was observed that older rabbits were less susceptible to the infection than younger ones. Fairly young animals, 12 to 16 weeks of age, were always used.

Fig. 2A shows a photograph of 2 wounds on one side of a rabbit which had been inoculated 3 days before. Thirty such wounds had an average diameter of 9.5 ± 0.8 mm, and healed in 17-20 days when treated with 5% sulfathiazole in methocel. The infections became much more involved with time and gradually

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[2] Nungester, W. J., Jourdonais, L. F., and Wolf, A. A., *J. Infect. Dis.*, 1936, **59**, 11.

‖ We wish to thank Doctor David Klein of the Wilson Laboratories, Chicago, Ill., for the sample of granular mucin "Type 1701-W" which was supplied.

[3] Parker, J. T., *J. Exp. Med.*, 1924, **40**, 761.

¶ We wish to acknowledge the cooperation of our colleague, Dr. E. A. Strakosch, Div. of Dermatol. and Syphilol., University of Minnesota, for supplying these strains, one of which proved to be sulfonamide-resistant.

** We wish to thank Dr. Nicholson for his kindness in sending us this strain of staphylococcus.

[4] Jackson, S. H., Nicholson, T. F., and Holman, W. L., *J. Path. Bact.*, 1940, **50**, 1.

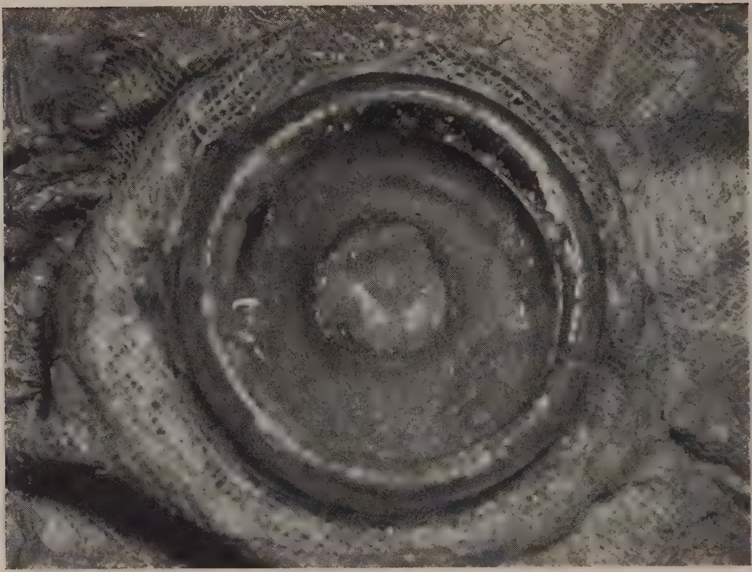

FIG. 2B.
Photograph of infected wound, 7 days after inoculation.

became more undermining and ulcerative after a week or more. Fig. 2A is shown to illustrate the appearance and uniformity of the wound dimensions and infection, and Fig. 2B represents an infected wound after 7 days duration.

Smears and gram strains were made from the wounds each time they were dressed in order to ascertain whether the infection was still associated with the causative organism, and broth cultures were made once weekly for a qualitative bacteriological test. In all cases, the wounds yielded pure cultures of *Staphylococcus aureus*.

Jensen[5] has suggested that the virulence of staphylococci could be maintained constant by lyophilizing them on glass beads or in vials to be used for subcultures for the wound inocula as desired. This probably would be as satisfactory, if not better, than the method of repeated passage from wound to wound. The lyophilization method is being investigated at present.

Summary. This report describes a method of producing and dressing open skin wounds so that a uniform standardly infected wound can be made for controlled topical chemotherapy studies, with healing time by secondary intention used as the main criterion of the effectiveness of the therapy. If desired, as described in the previous paper,[1] histological studies of epithelization and granulation can be made on biopsied wounds, in addition to the determination of healing time.

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[5] Jensen, N. K., personal communication.

14074 P

Estimation of the Third Component (C′3) of Complement.*

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The third component of complement (C′3) has been defined in terms of its role in a hemolytic system. It is also known as the component which is inactivated by cobra venom or by yeast. In an attempt to discover which fraction of yeast inactivated C′3, Pillemer and Ecker[1] succeeded in isolating an insoluble carbohydrate from yeast which destroys or removes the activity of C′3 in amounts vastly smaller than the whole yeast or the zymin preparation of Whitehead, Gordon and Wormall.[2] For the sake of simplicity this insoluble carbohydrate will henceforth be called *zymosan*.[†] It was suggested that the inactivation of complement by yeast was due to the adsorption of C′3 on this carbohydrate. In a subsequent paper Ecker, Jones and Kuehn[3] noted that in the presence of 10% NaCl zymin did not inactivate C′3. Likewise, it has since been found that 10% NaCl suppresses the adsorption of C′3 by zymosan.

In the light of these facts it was therefore proposed to study the adsorbate with regard to the uptake of nitrogen. A stock suspension of the zymosan was made by placing it in saline (0.9%) in the proportion of 1 mg per ml. This lot of carbohydrate contained 3.14% N. Five ml of this zymosan suspension was added to each of a series of tubes, centrifugalized, and the supernates discarded. Varying amounts of pooled guinea pig serum were then added (in duplicate) to tubes containing the zymosan. Making use of the fact that the zymosan did not inactivate C′3 in the presence of 10% NaCl, duplicate tubes containing enough solid NaCl to make 10% were run parallel to each given quantity of serum. Rubber stoppers were inserted into the tubes which were then thoroughly shaken and incubated in a water bath at 37°C for one hour. Shaking was repeated every 10 minutes during the incubation period. Subsequently all tubes were centrifugalized and the precipitates washed 3 times with cold saline (0.9%) before their quantitative transfer to micro-Kjeldahl flasks. The supernatant serum was retested in one- and 2-unit aliquots for any residual hemolytic activity by the method of Ecker and Pillemer.[4] The sera containing 10% NaCl were restored to isotonicity by dilution with distilled water previous to titration. Table I shows that the serum in the tubes with 10% NaCl was not inactivated by the zymosan. The amount of zymosan chosen was sufficient to inactivate the serum in all quantities except the highest, namely, 8 ml. At this volume about 20% of the original activity was retained. When the N contained in the zymosan is subtracted from that of the entire adsorbate and expressed per ml of serum, a constant amount of N is not obtained. However, when the N contained in the parallel tubes with 10% NaCl is subtracted from that of the total N obtained from the untreated serum, an approximately constant figure is obtained.

The data further show that as the volume of serum is increased per constant amount of zymosan, the total amount of N taken up per ml of serum decreases rapidly to a critical level, at which point C′3 begins to remain in the supernate. Table I shows (third column from right) that more than C′3 is removed

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* Aided by a grant from the Commonwealth Fund.

[1] Pillemer, L., and Ecker, E. E., *J. Biol. Chem.*, 1941, **137**, 141.

[2] Whitehead, H. R., Gordon, J., and Wormall, A., *Biochem. J.*, 1925, **19**, 618.

[†] This name for the insoluble carbohydrate was chosen to indicate its source from yeast and its general carbohydrate character.

[3] Ecker, E. E. Jones, C. B., and Kuehn, A. O., *J. Immunol.*, 1941, **40**, 81.

[4] Ecker, E. E., and Pillemer, L., *Annals N. Y. Acad. Sci.*, 1942, **43**, 63.

TABLE I.
Nitrogen Determination* of the Zymosan Adsorbate.

Zymosan, mg	Guinea pig serum, ml	NaCl 10%	% activity remaining Units 1	2	Total N, mg	Total N less Zymosan N: Per given vol. serum, mg	Per ml serum, mg	Total N less NaCl blank—"C′3 N": Per given vol. serum, mg	Per ml serum, mg
5	1	0	0	0	.200	.043	.043	.025	.025
5	1	+	90	100	.175	.018	.018		
5	2	0	0	0	.220	.063	.032	.037	.019
5	2	+	90	100	.183	.026	.013		
5	4	0	0	0	.261	.104	.026	.081	.020
5	4	+	95	100	.180	.023	.006		
5	8	0	20	40	.328	.171	.021	.147	.018
5	8	+	95	100	.181	.024	.003		
5	0	0			.157				

*Average of duplicate.

from the serum when an excessive amount of zymosan is used. For instance, using 1 ml of serum with 5 mg of the zymosan a total of 0.043 mg N is taken up, but when 4 ml is employed with the same amount of zymosan, the N uptake decreases to 0.026 mg per ml of serum. A volume of 8 ml exceeds the capacity of the zymosan to remove all the C′3, and some of the third component remains free; hence the C′3 N comprises a little more than 0.021 mg per ml. This value compares favorably with those of the last column in which it is found that although C′3 is suppressed in the presence of 10% NaCl, some other material is taken up. The assumption is made in the last column that this additional N can be used as a blank to subtract from the total N adding from untreated serum; the difference is presumably "C′3 N." Nonetheless, it must be stressed that an average of about 0.02 mg N per ml represents only a *maximum* value for C′3, because an excess of the zymosan removes other material from serum besides C′3. This phenomenon was also noted in the case of zymin when an excess was used.[3]

Human serum was studied in the same manner as guinea pig serum, and similar values were found. In addition, attempts at varying experimental conditions in the case of human serum prior to the determination of N gave some interesting results. It was observed that when the same serum sample was treated with constant amounts of the zymosan that the total amount of N adsorbed after the first treatment was the same regardless of the volume of serum employed (2 to 8 ml). Two percent NaCl was found to be sufficient to suppress the inactivation of C′3 by the zymosan. The total N "adding" from heated serum (56° for 30 minutes) was not consistent. Human serum diluted as much as 1:5 was inactivated by constant amounts of the zymosan and as much N was added as from the original volume of undiluted serum. Previous treatment of the zymosan with 0.1 N NaOH and heating in a boiling water bath for 30 minutes practically destroyed its C′3 inactivating capacity. Furthermore, a preliminary study now going on indicates that acidification of the serum to a pH of 5.4 inhibits the removal of C′3 provided a minimum amount of the zymosan is employed. Experiments on elution of C′3 also are in progress.

14075

Development of Sulfathiazole-Resistant Gonococci *In vitro.*

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Gonococci cultivated in gradually increasing concentrations of sulfanilamide or sulfapyridine readily develop resistance, or fastness, to the bacteriostatic action of these sulfonamides.[1,2] With sulfathiazole, however, attempts to produce resistant strains of gonococci have not been successful.[3] The potential clinical significance of this observation has been pointed out in a recent editorial in the *Journal of the American Medical Association* as follows, "This failure of the gonococcus to develop resistance to sulfathiazole suggests that sulfathiazole-fast strains are not likely to be developed in the clinic or to be spread to the general population."[4]

Recent experiments in this laboratory have indicated that under proper conditions organisms susceptible to the bacteriostatic action of any of the sulfonamides should be capable of becoming resistant to all of the sulfonamides.[5] If this theory is correct, it should be possible to develop sulfathiazole-resistant gonococci, and the following *in vitro* experiments were undertaken to determine whether this could be accomplished.

Materials and Methods. A strain of *Neisseria gonorrhoeæ* isolated from a male with gonococcal urethritis was employed for the experiments. Using the quantitative technic previously described,[5] a constant inoculum of about one million organisms per cc was transferred every 5 to 7 days in 20% ascitic broth containing gradually increasing concentrations of sulfonamides, beginning with sulfanilamide 25 μg per cc and sulfathiazole 0.025 μg per cc. After 3 transfers the sulfonamide concentrations were doubled, and this process was repeated until the concentration of sulfanilamide was 500 μg per cc, and that of sulfathiazole was 10 μg per cc. Growth of the control and resistant organisms was measured by turbidity readings on the Coleman Universal Spectrophotometer. Using the same technic, measurements were made of the bacteriostatic effect of sulfapyridine and sulfadiazine on the control and resistant organisms.

Results. Growth curves of the control and resistant organisms are presented in Fig. 1. It is evident that transfers in gradually increasing concentrations of sulfonamides caused the gonococci to become markedly resistant to both sulfanilamide and sulfathiazole. These same sulfonamide concentrations completely inhibited the growth of the control organisms; indeed, they were completely inhibited by concentrations less than one-eighth as great as those shown in the figure, even after 30 days.

TABLE I.

The Bacteriostatic Action of Four Sulfonamides upon Sulfathiazole-Resistant Gonococci. Growth is expressed in terms of optical density (Turbidity). See text for explanation.

Drugs	μg/cc	Control organisms	Sulfathiazole-resistant gonococci
Sulfanilamide	500	0	.36
Sulfapyridine	50	0	.35
Sulfadiazine	10	0	.32
Sulfathiazole	10	0	.30

Table I shows the results when the sulfathiazole-resistant organisms were grown for 5 days in media containing sulfathiazole 10 μg per cc, sulfadiazine 10 μg per cc, sulfapyridine 50 μg per cc, and sulfanilamide 500 μg per cc. Good growth occurred with all the sulfon-

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[1] Boak, R. A., Charles, R. L., and Carpenter, C. M., Pub. No. 11, American Society for the Advancement of Science, Lancaster, Pa. The Science Press, 1939, p. 118.

[2] Westphal, L., Charles, R. L., and Carpenter, C. M., *Ven. Dis. Inf.*, 1940, **21**, 183.

[3] Carpenter, C. M., Charles, R., and Allison, S. D., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 527.

[4] Editorial, Sulfanilamide Fast Gonococci, *J. A. M. A.*, 1942, **119**, 31.

[5] Kirby, W. M. M., and Rantz, L. A., *J. Exp. Med.*, 1943, **77**, 29.

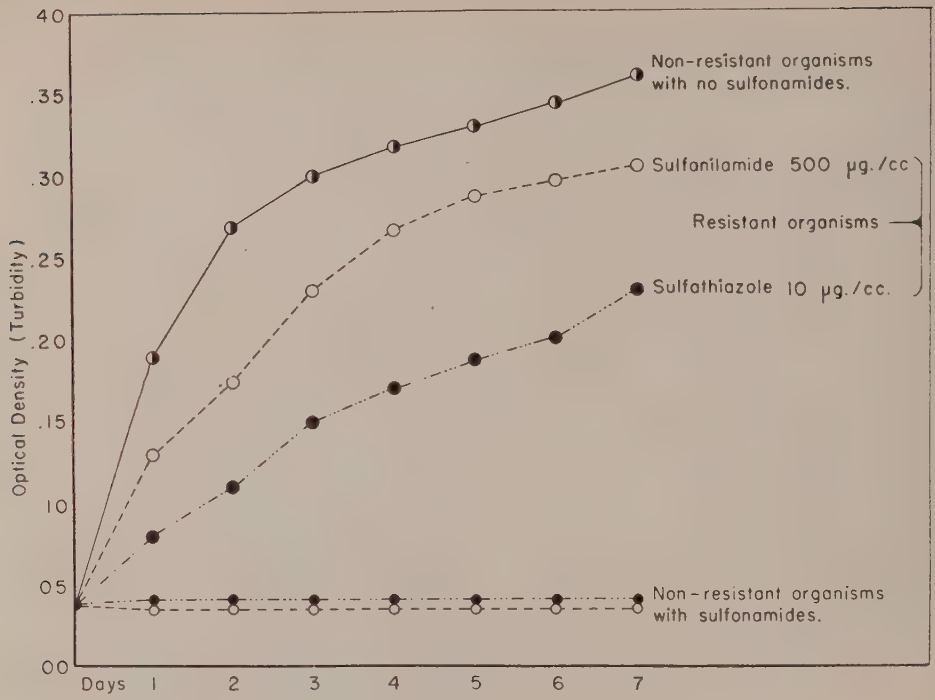

Figure I. Growth Curves of Resistant and Non-resistant Gonococci.

amides, while the controls were completely inhibited. The results when the sulfanilamide-fast gonococci were grown in media containing the other three sulfonamides were essentially the same, and therefore are not included in the table.

Comment. In these experiments sulfathiazole-resistant gonococci have been developed with comparative ease. The failure of previous workers to accomplish this may have been due to beginning with too high a concentration of sulfathiazole, for it must be realized that in these *in vitro* experiments the bacteriostatic potency of sulfathiazole is more than 50 times as great as that of sulfanilamide.

The sulfathiazole-resistant gonococci were also resistant to sulfanilamide, sulfapyridine, and sulfadiazine, and the sulfanilamide-resistant organisms were also resistant to sulfapyridine, sulfadiazine, and sulfathiazole. These findings, which are being extended to other strains of the gonococcus, are in accord with the previously advanced thesis[5] that the development of sulfonamide resistance represents an interaction between the organisms and the *p*-amino nucleus of the sulfonamides, and that organisms susceptible to the bacteriostatic action of any of the sulfonamides are capable of becoming resistant to all of the sulfonamides.

Summary and Conclusions. Using a simple *in vitro* technic, it has been possible to develop both sulfanilamide-resistant and sulfathiazole-resistant gonococci. The sulfathiazole-resistant organisms were resistant to sulfanilamide, sulfapyridine, and sulfadiazine, and the sulfanilamide-resistant organisms were also resistant to sulfapyridine, sulfadiazine, and sulfathiazole. The significance of these observations is discussed.

14076

Purification of Equine Encephalomyelitis Virus by Ultracentrifugation and Maintenance of Its Activity with Cysteine.

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Purification of enzymes[1] and viruses[2] is aided by quantitative methods of determining the activity and solids in the various fractions.

It has been reported[3] that the virus of equine encephalomyelitis has been concentrated and at least partially purified, but quantitative data showing the results of the various steps have not been published. We have carried out experiments designed to separate the virus from non-virus material by using the following procedures: (a) ultracentrifugation, (b) fractional precipitation by dialysis or dilution, (c) fractional precipitation by heparin. Since ultracentrifugation was the only procedure to yield consistent and desirable results as regards purification, discussion of the other experiments will be brief.

The results of a number of ultracentrifugation experiments are shown in Tables I and II. It may be seen from these tables that ultracentrifugation forces most of the virus into the pellet and that the titer per gram of nitrogen of the resuspended pellet is about 10 times higher than the starting material.

Since one of the major difficulties in purification of this virus is the maintenance of activity, we have also studied the effect of certain reducing agents on the stability of ultracentrifuge purified virus suspensions. The results of this study (Table III and Fig. 1) show that at pH 7.0-8.0 in the presence of M/10 cysteine the activity of ultracentrifuge-purified virus is relatively constant.

TABLE III.

Experimental procedure: To 2 ml of ultracentrifuge purified virus was added 2 ml of 0.2 molar reducing reagent in phosphate buffer so the final pH was 6.8-7.8. The thioglycollate was prepared by mixing solutions of calcium thioglycollate with Na_2HPO_4 and centrifuging off the precipitate.

	Original	8 days	15 days	100 days
Control	$10^{-7.8}$	$<10^{5.5}$	$10^{-2.3}$	
Cysteine		$10^{7.3}$	$10^{7.8}$	$10^{5.7}$
Hydroquinone		$<10^{5.5}$	$<10^{2.5}$	
Thioglycollate		$<10^{5.5}$	$<10^{1.5}$	

Experimental. Virus activity was followed by use of the 50% mortality endpoint of 10-day chick embryos, which allows frequent accurate titrations of activity.[4]

Our investigation was limited to the eastern variety of the virus, isolated in this laboratory from horses[5] in 1933. The solution of virus used as starting material was prepared as follows: 10-day chick embryos were infected by inoculating the chorioallantoic membrane with 1 drop of 1/100 dilution of infected guinea pig brain. The embryos were harvested at 23 hours, ground in a Waring Blendor in cold saline solution, buffered with phosphate to make a 20% suspension by weight. This suspension was cleared of large particles by centrifugation in a Swedish angle centrifuge at 4,000 R.P.M. for 20 minutes. Serial tenfold dilutions in cold buffered saline solution were made and each of 3 or 4 dilutions, which were likely to fall on either side of the 50% endpoint, was then inoculated on five 10-day embryos. The 50% mortality endpoint was calculated[6] after the eggs had been incubated 3 days.

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[1] Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.

[2] Stanley, W. M., *Science*, 1935, **81**, 644.

[3] Sharp, D. G., Taylor, A. R., Beard, D., Finkelstein, H., and Beard, J. W., *Science*, 1940, **92**, 359; Taylor, A. R., Finkelstein, H., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 462; Taylor, A. R., Sharp, D. G., Finkelstein, H., Beard, D., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 648.

[4] Bang, F. B., *J. Exp. Med.*, 1943, **77**, 337.

[5] TenBroeck, C., and Merrill, M. H., Proc. Soc. Exp. Biol. and Med., 1933, **31**, 217.

[6] Reed, L. J., and Meunch, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.

		Titer*			
No. of centrifugings	Time of runs min	Original	Pellets resuspended in same vol.	First supernatant	Change in titer
2	75	8.0	8.0		0
2	45	8.0	6.4	4.3	—1.6
3	30	7.5	7.8	6.0	+0.3
2	45, 30	7.5	8.0	5.5	+0.5
2	45, 30	8.5	7.8	4.2	—0.7
1	45	8.4	8.0		—0.4
2	45, 30	8.2	7.6		—0.6
2	30	8.0	7.8		—0.2
2	45	7.5	7.6	4.4	+0.1

*The figures shown below are exponents of 10. They represent the serial tenfold dilution of the original solution to produce 50% mortality.

TABLE II.

	Original suspension		Ultracentrifuged pellet resuspended		Titer/g nitrogen		
No. of centrifugings	Titer	Nitrogen, mg/ml	Titer	Nitrogen, mg/ml	Original suspension	Resuspended pellet	Purification
1	$10^{8.1}$	0.8*	$10^{8.1}$	0.06	$10^{11.2}$	$10^{12.8}$	13×
2	$10^{8.3}$	0.8	$10^{9.0}$	1.2	$10^{11.4}$	$10^{11.9}$	3×
3	$10^{7.5}$	0.8	$10^{9.2}$	0.6	$10^{10.6}$	$10^{12.4}$	60×
2	$10^{8.0}$	0.8	$10^{8.2}$	0.7	$10^{11.1}$	$10^{11.4}$	2×
2	$10^{7.6}$	0.7	$10^{7.8}$	0.1	$10^{10.8}$	$10^{11.8}$	10×

*This figure is based on a number of measurements made on suspensions prepared at other times which varied but slightly.

Ultracentrifugation. Table I presents the results of centrifugation of clarified 20% infected embryo suspensions in a Bauer and Pickels air-driven ultracentrifuge[7] spun at 30,000 R.P.M. Since the tubes are at an angle this speed produces 50,000-90,000 times gravity. The centrifuge head was precooled to 5°C. In these experiments the pellets so obtained were resuspended in cold normal buffered saline, cleared in the Swedish centrifuge at 4,000 R.P.M. and then immediately titered by serial tenfold dilutions or ultracentrifuged again.

The results in Table I show clearly that the virus is thrown down in the pellet and that it may be recovered from the pellet by resuspension in buffered saline. Previous experiments have shown that the titer of one solution when run in duplicate may vary[4] as much as $10^{0.6}$ and this error must be taken into account in appraising the above figures. The average loss in these experiments (with one low result excluded) is $10^{0.1}$. This indicates that most of the virus is recovered.

Since, as may be seen from Table I, most of the original virus activity is in the resuspended residue, while a fair proportion of the original protein is left in the supernatant liquid from the first ultracentrifugation, it follows that there has been at least a partial purification. This is seen more directly from the columns of Table II showing the virus activity per gram of nitrogen.

Fractional Precipitation. Precipitation was carried out by dialysis in a rocking dialyzer against distilled water for 36 hours. Ultracentrifuge purified virus was treated in this way but the results were inconstant. A variation of this method of precipitation was carried out by diluting the purified concentrates in distilled water, then centrifuging out the precipitate. This too yielded inconstant results, although individual experiments did show a significantly greater amount of activity of protein nitrogen in the precipitate.

Certain macromolecules, including plant

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[7] Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge,* Oxford Press, 1940.

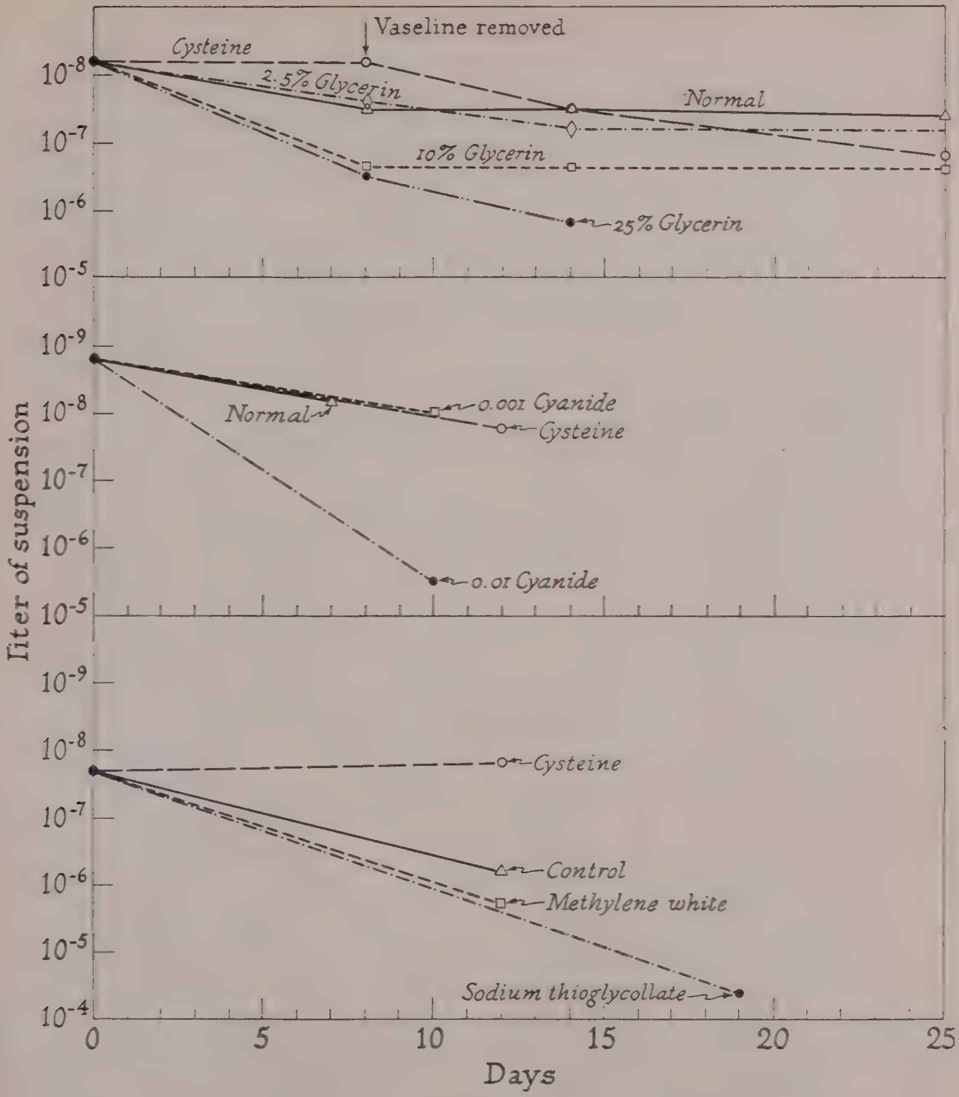

FIG. 1.

viruses, may be separated from solutions by adding heparin to make a 5% solution.[8] This was attempted on freshly prepared ultracentrifuged concentrates of equine encephalomyelitis and the resulting precipitate was then thrown down by low speed centrifugation. Titration of the precipitate and of the supernatant again showed no concentration of virus in the precipitate.

Preservation of Virus Activity. The previous work was made difficult by a frequent loss of activity of the virus during manipulation. This was particularly apparent when working with suspensions partially purified by centrifugation. For this reason we turned to a study of the factors affecting the stability of such suspensions. Other workers[9] have found that the stability is unaffected by a relatively wide pH range. Certain enzymes are active only within certain oxidation-reduction ranges.[10] Among viruses, that of tomato spotted wilt is highly susceptible to oxidation by air and may be protected by reducing agents such as cysteine and sodium thioglycollate.[11] Mueller found that the virus of Rous's sarcoma may be protected against oxidation by the addition of cysteine.[12]

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[8] Cohen, S. S., *J. Biol. Chem.*, 1942, **144**, 353.

Zinsser and Tang[13] have found that cysteine stabilizes herpes virus.

The results in Table III and Fig. 1 show the effect of certain reducing agents under vaseline and of glycerine in several concentrations on the stability of virus. Unless otherwise indicated the virus was ultracentrifuge purified; the pH was held at 7.0-7.6 with M/10 phosphate; the concentration of reducing agent was M/10; and the temperature was 5-10°C. Methylene white was prepared by reducing methylene blue with metallic zinc and hydrochloric acid followed by rapid neutralization and centrifugation of the precipitate. The supernatant was used. Cyanide was used in two instances to destroy some cellular enzymes which might be present and causing the inactivation. In several instances after the experiments were finished there was still some active reducing agent as detected with methylene blue.

In the experiments shown in Table III the temperature accidentally rose to something above 10°C. As a result of this accident, however, the stabilizing effect of cysteine was forcibly brought to our attention for there was only a perceptible drop of virus activity in the tube containing cysteine but a marked drop in all others including the control.

Summary. The virus of Eastern equine encephalomyelitis may be concentrated and partially purified by ultracentrifugation. M/10 cysteine retards spontaneous inactivation of the ultracentrifuge purified virus.

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[9] Finkelstein, H., Marx, W., Bridgers, W. H., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 103; Finkelstein, H., Marx, W., Beard, D., and Beard, J. W., *J. Inf. Dis.*, 1940, **66**, 117.

[10] Hellerman, L., *Cold Spring Harbor Symp. Quant. Biol.*, 1939, **7**, 165.

[11] Best, R., *Aust. J. Biol. and Med. Sci.*, 1939, **17**, 1.

[12] Mueller, J. H., *J. Exp. Med.*, 1928, **48**, 343.

[13] Zinsser, H., and Tang, F. F., *J. Immunol.*, 1929, **17**, 343.

14077

Action of Promin and Diamino-Diphenyl Sulfone upon Tubercle Bacilli; Antipromin Action of p-Aminobenzoic Acid.

WM. STEENKEN, JR., AND FRED H. HEISE. (Introduced by L. U. Gardner.)

From Research and Clinical Laboratory, Trudeau Sanatorium, Trudeau, N.Y.

A series of test tube experiments was undertaken to determine the action of promin* and its parent substance, diamino-diphenyl sulfone, upon the tubercle bacillus, and to learn whether or not *p*-aminobenzoic acid would inhibit the effect of these compounds as it has been shown to inhibit the effect of sulfanilamide upon other organisms.

All these tests were made upon both virulent and avirulent mutants of the human strain H_{37} used by Feldman, Hinshaw and Moses,[1-4]

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* Promin used in this study was kindly supplied through the courtesy of Dr. E. A. Sharp, Parke, Davis and Company, Detroit, Mich.

[1] Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Proc. Staff Meet., Mayo Clin.*, 1940, **15**, 695.

[2] Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Ibid.*, 1941, **16**, 187.

[3] Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Am. Rev. Tuberc.*, 1941, **45**, 303.

[4] Feldman, W. H., Mann, F. C., and Hinshaw, H. C., *Ibid.*, 1942, **46**, 187.

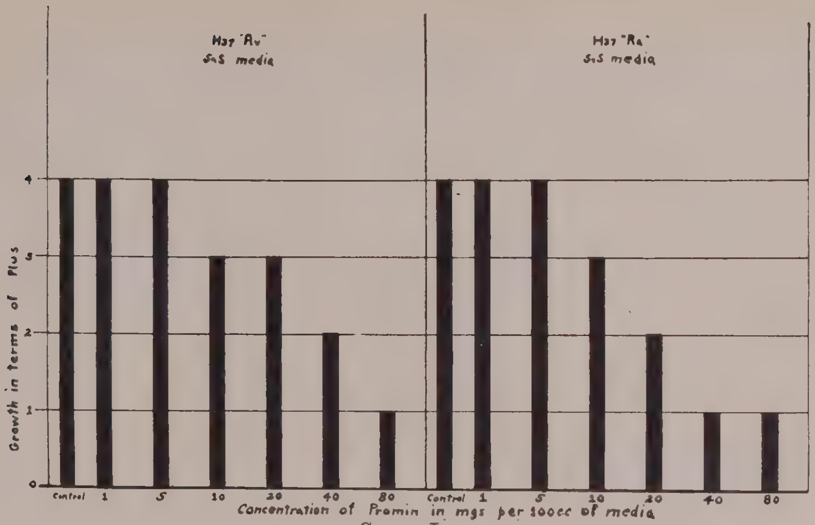

CHART I.

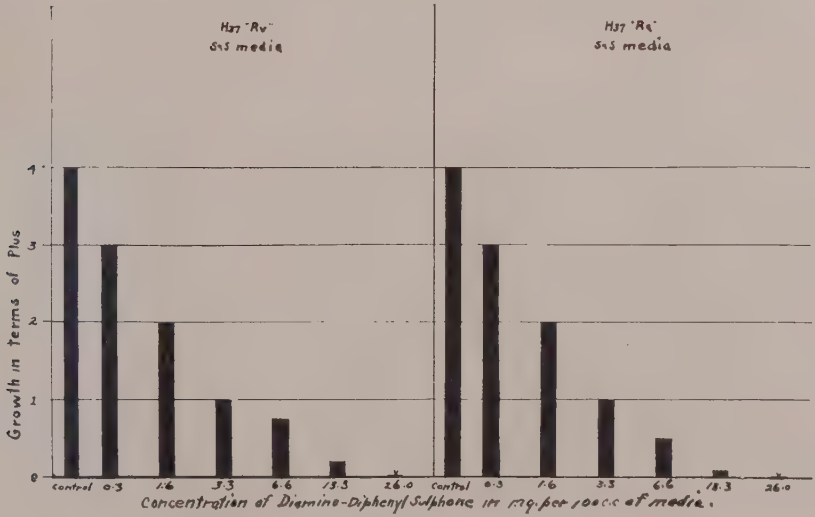

CHART II.

in their work on promin, and by writers in previous studies on sulfonamide drugs. The cultures were grown on Steenken and Smith's solid medium, or on Proskauer and Beck's fluid medium.

Promin in Steenken and Smith's Medium. Promin was added to tubes of this medium in concentrations from 1 to 80 mg% and the mixtures were then seeded with the 2 variants of the standard bacillus. Chart I demonstrates comparatively slight inhibitory action until concentration of 40 mg% is attained. The effect is more marked upon the avirulent (R_a) mutant.

Diamino-Diphenyl Sulfone[†] *in Steenken*

[†] Diamino-diphenyl sulfone was kindly supplied by Abbott Laboratories, North Chicago, Ill.

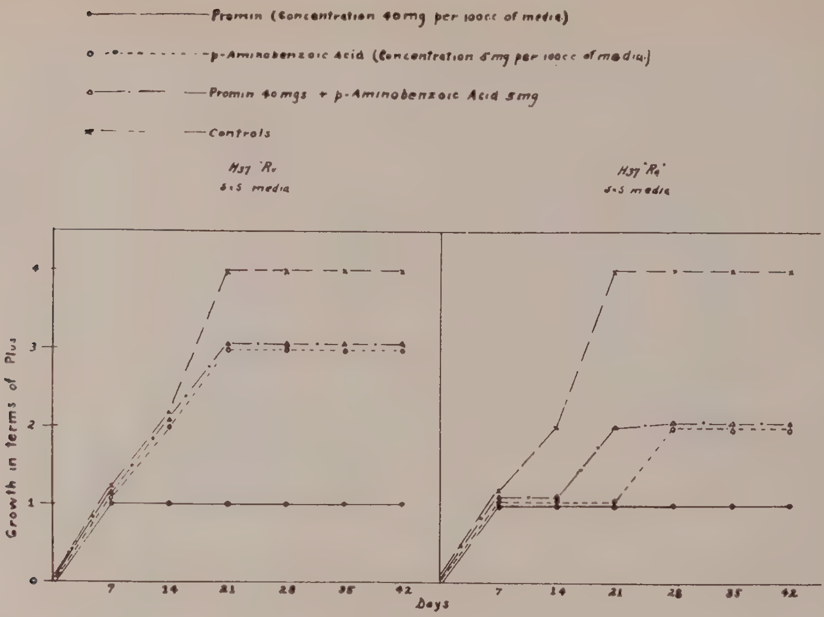

CHART III.

and Smith's Medium. The quantities of this drug used were based on the amount of the parent substance, diamino-diphenyl sulfone, which constitutes about one-third of the molecular weight of promin. Therefore the weights used in this test were in the ratio of one part of the diamino-diphenyl sulfone as compared to 3 parts of promin used in the previous test. These quantities varied from 0.3 to 26 mg% as shown in Chart II. These drug concentrations had much more effect upon the growth of the tubercle bacilli than the larger quantities by weight of promin. The additions of 1.6 mg% reduced growth to the same degree produced by 40 mg% of promin.

P-Aminobenzoic Acid and Promin in Proskauer and Beck's Fluid Medium. These tests involved observations upon the action of this substance alone and in combination with 40 mg% of promin, together with appropriate controls. The results are plotted in Chart III on the basis of the amount of growth observed at intervals during 42 days after planting the cultures. It will be noted that the untreated control medium produces maximum growth after 21 days and that promin alone limits bacterial growth to a low level which does not increase appreciably after the first week.

The addition of *p*-aminobenzoic acid in a concentration of 5 mg% exerts a moderate degree of inhibition of growth, and this effect is not appreciably increased by the simultaneous presence of 40 mg% of promin. The result was checked further by determining the weight of organisms produced in 4 weeks on Proskauer and Beck's fluid medium to which the test drugs had been added in the concentrations just mentioned.

Chart IV shows that the results were comparable to those based upon an estimate of the extent of the growth. Promin alone kept the growth down to very low levels. In the presence of *p*-aminobenzoic acid, promin caused only slightly less inhibition of growth than *p*-aminobenzoic acid alone.

In both the last 2 groups of tests the effects were somewhat more marked upon the avirulent dissociant R_a of the H_{37} strain.

Discussion. Our experiments indicate that the concentration of promin required to produce inhibition of growth of tubercle bacilli (H_{37} R_v) *in vitro,* is far greater than the concentration required to retard the progres-

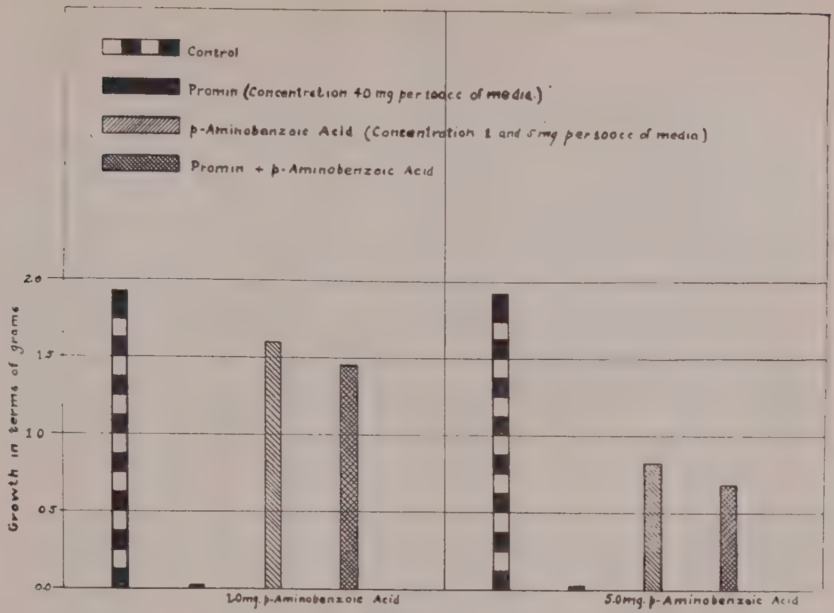

CHART IV.

sion of the disease in the animal body as reported by Feldman, Hinshaw and Moses.

Apparently, the action of promin in the animal body (guinea pig) is quite similar to the action of other sulfonamides against other microörganisms, in that they produce a bacteriostatic and not a killing effect upon the microörganisms.

Diamino-diphenyl sulfone produces a much greater inhibition of growth upon the H_{37} (R_v and R_a) microörganisms when added to media on the calculated basis of the available amount of the parent substance diamino-diphenyl sulfone present in promin, which is about one-third of the molecular weight of promin.

On the basis of this finding it is possible that the parent substance diamino-diphenyl sulfone may be equally, or more, effective than promin against tuberculous disease in guinea pigs.

From these studies it can be seen that *p*-aminobenzoic acid exerts a marked anti-promin effect upon the growth of the tubercle bacilli. This action is similar to the anti-sulfonamide action of *p*-aminobenzoic acid upon the growth of other bacteria as described by Woods.[5]

It might also be suggested that *p*-aminobenzoic acid be added to the cultures when culturing for tubercle bacilli from wounds treated with sulfonamides. Since the drugs are usually applied as powder or a thick paste, the drug in such form is in a concentration that would retard or inhibit the growth of the tubercle bacillus.

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[5] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

14078 P

Effect of Organic Nitrates on Coronary Flow.*

Margaret Wrightington. (Introduced by H. C. Hodge.)

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Erythrol tetranitrate and mannitol hexanitrate,[†] since their introduction by Bradbury,[1] have been used to some extent in the prophylactic treatment of patients with angina pectoris; and glycerol trinitrate has received extensive study both experimentally[2] and clinically.[3] This study was undertaken to determine the comparative effects of these 3 drugs on the coronary flow through the isolated rabbit heart.

Method. A modified Langendorf preparation of young and vigorous rabbit hearts was used. The hearts were perfused with Locke's solution containing 1% dog serum,[4] and 0.1% glycine[5] and adjusted to a pH of 7.4. A constant perfusion pressure of 60 cm of

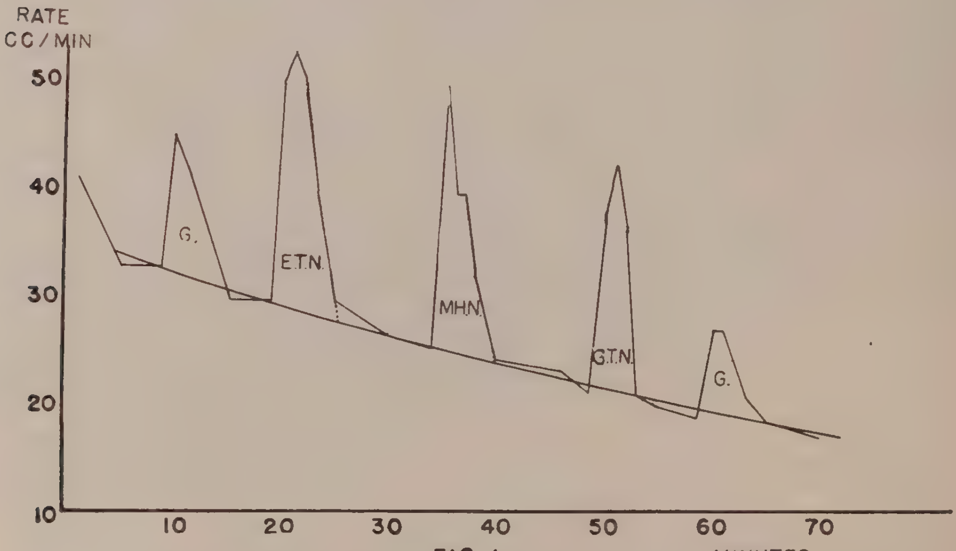

FIG. 1

Graph of Coronary Flow Rate. A typical experiment showing the increase in flow produced by the 3 nitrates with a glycerine control at the beginning and the end. G = glycerine, E.T.N. = erythrol tetranitrate, M.H.N. = mannitol hexanitrate, and G.T.N. = glycerol trinitrate. Base line is indicated.

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* Aided by a grant from the R. J. Strasenburg Company.

[†] Due to the chemical instability of mannitol hexanitrate, this compound was obtained in the form of maxitate, a stabilized preparation furnished by the R. J. Strasenburg Company, and was extracted with ether and suspended in glycerine. A suspension seemed necessary since it was so insoluble in non-toxic solvents.

[1] Bradbury, J. B., *Brit. Med. J.*, 1895, **2**, 1213.

[2] Katz, L. N., Lindner, E., Weinstein, W., *Arch. Internat. de Pharmacodyn. de Therap.*, 1938, **59**, 399.

[3] Riseman, J. E. F., and Brown, M. G., *Arch. Int. Med.*, 1937, **60**, 100.

[4] Wedd, A. M., *J. Pharm. and Exp. Therap.*, 1936, **57**, 179.

[5] Hermann, G., Dechard, G., and Erhard, P., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 547.

TABLE I.
Average Net Increase in Volume and Rate of Coronary Flow* Produced by Various Nitrates.

	Avg area under curve, cm^2	Standard deviation	Avg rate, cc/min	Standard deviation
Mannitol Hexanitrate	.29	.09	.044	.020
Erythrol Tetranitrate	.31	.21	.040	.030
Glycerol Trinitrate	.25	.16	.036	.024

*The area under the curve represents the volume of the increase in flow over the base line values. The average increase in rate is the area divided by the duration of the increase. The net values represent the increase over the control glycerine curves. These data represent averages from 17 experiments.

water and a constant temperature of 32°C were maintained throughout the experiment. The effects of solutions (a) of 40% erythrol tetranitrate and (b) of glycerol trinitrate in glycerine were compared to those produced by a fine suspension of 40% mannitol hexanitrate† in glycerine. Since there was no sharp drop in the coronary flow after the addition of the suspension it was believed that there was no demonstrable plugging of the capillaries. One milliliter of each of these solutions was injected over a period of 3 minutes into the perfusion fluid just above the heart. Only 3 drug tests were made on each heart; however, glycerine controls were run at the beginning and end of each experiment (Fig. 1). Since the activity of the heart and the rate of coronary flow gradually decreased both during these experiments and during control runs in which no drug was added, a base line was drawn along this fall. Comparative values for the 3 drugs were obtained by estimating with a planimeter the area of the increase above the base line following each drug. This determined the volume of the increment in coronary flow caused by the nitrate. Since the solvent, glycerine, produced some increase in the coronary flow, the net increase caused by each nitrate was determined by subtracting from the figure for each drug that for the glycerine curve next to it. For the second nitrate test an average of the 2 glycerine values was used.

Results. Erythrol tetranitrate, glycerol trinitrate and mannitol hexanitrate in these isolated hearts produced an increase in the flow of fluid through the coronaries which varied from 20 to 60%. The response differed considerably from heart to heart, and as a rule varied directly with the vigor and activity of the individual hearts. The maintenance of a regular heart rate by rhythmic electrical stimulation in 9 experiments did little to reduce this variability. However, in each separate heart the response to each of the 3 drugs was of the same order of magnitude, and any effect of the spontaneous decrease in cardiac activity upon the coronary response was obviated by injecting the nitrates in different order in different experiments. The slight differences between the effects of the 3 nitrates shown in their average volume and rate increments (Table I) are not significant.

The author wishes to thank Dr. H. C. Hodge for his helpful criticism.

14079

Oral Glucose Tolerance Tests in Dogs with Intestinal Resections.

MARTIN G. GOLDNER AND ALF T. HAEREM. (Introduced by George F. Dick.)

From the Department of Medicine, The University of Chicago.

The oral glucose tolerance test depends not only on the state of the liver and pancreas but also on the absorption of the glucose from the gastro-intestinal tract. It is of interest, therefore, to know how resections of various parts of the gastro-intestinal tract influence the shape of the normal glucose tolerance curve.

Material. We had in our laboratory 6 dogs which had been operated upon more than 10 months ago and which were in good nutritional state and were kept on high caloric food rich in carbohydrate. Three of these dogs had undergone total gastrectomy; in 2 of these 3 dogs the major part of the jejunum or the ileum, respectively, had been removed in a second operation. In one dog jejunectomy and in 2 dogs ileectomy had been performed.

Test Procedure. The dogs, after a 12-15 hours' fast, were given the test meal through the stomach tube and the sugar content of the venous blood was estimated at fasting and 30′, 60′, 120′ and 180′ after ingestion of a 20% glucose solution. 1.5 g glucose was given per kg of body weight. The Miller-Van Slyke Mikromethod for blood sugar estimation was used. Three tests at intervals of one week were performed in each dog. As control, intravenous tolerance tests with 10 cc of a 50% glucose solution were performed which in all instances showed normal curves.

Results. Fig. 1 and Table 1 represent the various types of curves obtained. In the figure, the sketch at the left of each curve indicates the resection which had been performed. The curves and values represent the average value of the 3 tests. It can be seen that in all instances where a gastrectomy (alone or together with another resection) had been performed, the curves show a very high peak but return to the fasting level with little or no delay. The high peak and the return appear to be delayed after jejunectomy. The blood sugar curve is normal after ileectomy.

Comment. These experiments show the importance of a normal function of the stomach for the normal oral glucose tolerance test. Under physiological conditions a hypertonic glucose solution, like the test meal, is diluted in the stomach and released into the duodenum and upper jejunum where it is absorbed almost completely.[1–4] The stomach and the ileum absorb only very little. In contrast to earlier observations,[5] it has been found that the rate of absorption from duodenum and jejunum depends on the concentration of the glucose

TABLE I.

	Avg venous blood sugar				
		After ingestion of glucose meal			
Dog	Fasting	½ hr	1 hr	2 hr	3 hr
Normal	88	110	132	100	82
Gastrectomy	84	205	189	80	76
Gastrectomy and jejunectomy	83	259	240	106	112
Gastrectomy and ileectomy	93	251	263	132	110
Jejunectomy	82	134	207	227	180
Ileectomy	86	100	124	102	85

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[1] Magee, H. E., and Reid, E., *J. Physiol.*, 1931, **73**, 163.

[2] Groen, J., *J. Clin. Invest.*, 1937, **16**, 245.

[3] Shay, H., Gershon-Cohen, F., Fels, S., and Munroe, F. L., *Am. J. Digest. Dis.*, 1940, **7**, 456.

[4] Karr, W. G., Abbott, W. O., Hoffmann, O. D., and Miller, T. S., *Am. J. Med. Sc.*, 1940, **200**, 524.

[5] Cori, F. C., *J. Biol. Chem.*, 1925, **66**, 691.

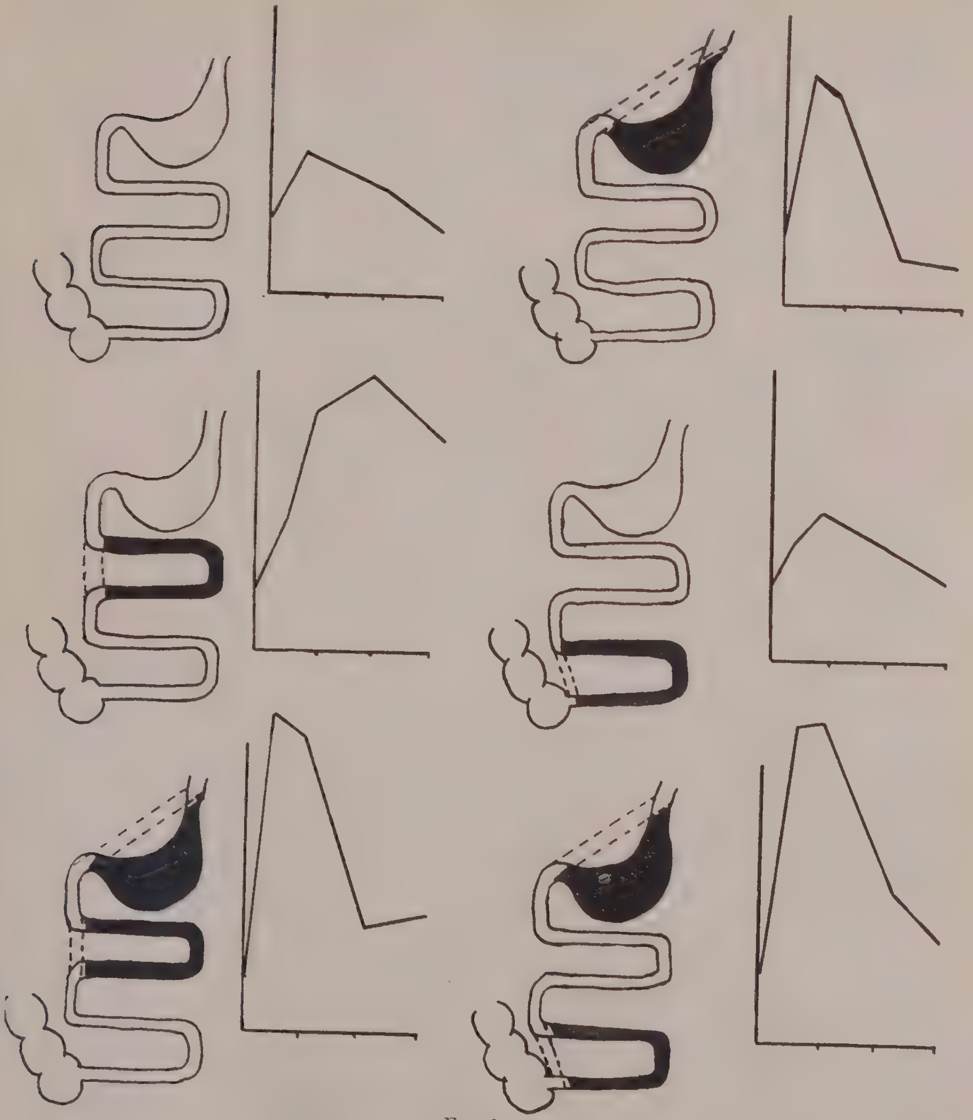

FIG. 1.

solution.[6–8] If in the case of gastrectomy the hypertonic solution enters the duodenum undiluted, glucose is absorbed at a higher speed. A higher blood sugar level is reached before the auto-regulation of the blood sugar begins. Since the ileum takes only little part in glucose absorption, ileectomy does not affect the normal oral glucose tolerance curve. The high and delayed curve after jejunectomy seems best explained by the assumption of interference with gastric and intestinal motility.

Abnormal oral glucose tolerance curves are

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[6] Abbott, W. O., Karr, W. S., Glenn, P. M., and Warren, R., *Am. J. Med. Sc.*, 1940, **200**, 532.

[7] Warren, R., Karr, W. G., Hoffmann, O. D., and Abbott, O. W., *Am. J. Med. Sc.*, 1940, **200**, 639.

[8] MacKay, E. M., and Clark, W. G., *Am. J. Physiol.*, 1941, **135**, 187.

obtained not infrequently in patients with organic or functional gastro-intestinal disorders.[9] Our experiments demonstrate that in such instances the disturbed gastro-intestinal absorption and motility plays an important role in determining the shape of the blood sugar curve.

Summary. After resection of the stomach or jejunum the oral glucose tolerance test in dogs shows a high and sometimes delayed curve; the curve is not altered after ileectomy.

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[9] Christlieb, W., *Dt. Arch. f. Klin. Med.*, 1937, **181**, 394.

14080

Detoxication of a Substituted Phenyl Stibonate in the Rat by Administration of *p*-Aminobenzoic Acid.

J. H. Sandground.* (Introduced by G. H. A. Clowes.)
From the Lilly Research Laboratories, Indianapolis, Indiana.

The writer has reported[1] experiments that demonstrated the highly protective powers of *p*-aminobenzoic acid when administered in substantial but well tolerated quantities to rats that simultaneously had received high lethal doses of Carbarsone, "Tryparsamide," arsanilic acid, and Acetarsone. Further experiments, to be published *in extenso* elsewhere, have shown that *p*-aminobenzoic acid provides equally high protection against other pentavalent arsenicals including phenyl arsonic acid and also against at least one of the trivalent arsenical antisyphilis drugs.

In view of the therapeutic value placed upon some of the less toxic antimony compounds in the treatment of such important tropical diseases as kala-azar and schistosomiasis, it seems desirable to record the capacity of *p*-aminobenzoic acid to protect rats against acute fatal doses of the German proprietary drug, "Stibosan" (sodium *m*-chloro *p*-acetyl amino phenyl stibonate), the only pentavalent antimony compound which was presently available for test purposes in our laboratory.

The test animals were albino rats, weighing approximately 100 g but otherwise unselected as to breed, sex, or age. Treated and control animals were maintained on a balanced diet in large communal cages. *p*-Aminobenzoic acid in the form of its water-soluble sodium salt was administered either by mouth or parenterally at the same time as the antimonial drug was introduced into the saphenous vein of the rat.

In the early stages of these studies, we administered a so-called sustaining dose of *p*-aminobenzoic acid for the following 2 or 3 days. Experiments already completed have indicated that the quantity of *p*-aminobenzoic acid administered has usually been more than was necessary to achieve the desired effect. A typical experiment is summarized in Table I.

Discussion. As tested in our laboratory against *Trypanosoma equiperdum* in rats, "Stibosan" has proved to have a very definite but relatively low therapeutic index. The quantity of drug necessary to effect a permanent cure of rats infected with *T. equiperdum* approaches the maximum dose that the majority of rats can tolerate. Since *p*-aminobenzoic acid interferes with the bacteriostatic action of sulfanilamide and its derivatives, it is of particular interest here to mention that *p*-aminobenzoic acid does not show any evidence of interfering with the trypanocidal potency of "Stibosan." This is shown by the disappearance of trypanosomes from the peripheral blood of rats that receive "Stibosan" plus large quantities of *p*-aminobenzoic acid. Owing to the already mentioned low trypanocidal efficacy of this antimonial com-

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* It is with great pleasure that the writer acknowledges the conscientious and invaluable technical aid he has received from Mr. Charles R. Hamilton in the prosecution of this study.

[1] Sandground, J. H., *Science*, 1943, **97**, 73.

TABLE I.
Protection of Rats Against Sodium *m*-chloro *p*-acetyl amino phenyl stibonate ("Stibosan").

Intravenous drug dose	Dose of *p*-aminobenzoic acid and frequency of administration	No. of rats		% survival	
		used in exp.	surviving	+ *p*-aminobenzoic acid	Control
300 mg/kg	1 g/kg x 3 days	12	12	100	
	controls	10	4		40
380 mg/kg	1 g/kg x 3 days	11	10	90	
	controls	12	0		0

pound, trypanosomes reappear in the blood of many of the treated animals and cause their death as late as 24 days after the start of the experiment.

The protective properties of *p*-aminobenzoic acid against "Stibosan" are spectacular. While the majority of protected rats remain normal in appearance and activity, the control rats receiving "Stibosan" alone rapidly lose weight, their fur becomes roughened and matted, they develop extreme flaccid paralysis of the hind quarters, and excessive secretion of the lachrymal glands seals the eyelids. Most animals die on the second to fifth days of the experiment. Lesions, especially of the kidney cortex, are outstanding in the morbid anatomy of fatal intoxication.

14081

Electrical Field of the Nerve Impulse.

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From the University of California at Los Angeles.

In a previous report, experiments were described purporting to test for the presence of an electro-magnetic field surrounding the segment of nerve bearing a nerve impulse.[1] Considerations arising from the bioelectric circuit hypothesis of nerve transmission lead to the conclusion that no electro-magnetic field should exist owing to the cancellation of the magnetic field accompanying the component of the circuit external to the axis cylinder by the magnetic field accompanying the component within the cylinder. The experiments indicated, in fact, that, within the sensitivity of the measuring instrument, no electro-magnetic field was present.

The passage of a nerve impulse involves, however, not only the existence of local bioelectric currents through the electrolytic media within and without the axis cylinder, but the existence of electrical lines of force which originate at any point of high potential and terminate at a point of lower potential. In the case of the nerve impulse the zone of depolarization will be characterized by a low electrical potential with respect to the 2 intact zones on either side of it. Hence there will be electrical lines of force originating in the two intact regions and terminating in the depolarized region. If a detecting instrument is interposed in the space containing these lines of force, then an electric charge is induced upon the instrument and may be detected with a suitable recorder.

A detector placed alongside a conducting nerve will convey to the recording instrument 2 peaks of such induced potential; one peak will occur when the depolarized region is approaching the detector, the other when it has passed under it and is receding from the detector. If, however, the nerve is crushed for a length of several centimeters and the detector is placed at the boundary of the intact and crushed region, then but one peak will be observed.

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[1] Gengerelli, J. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 189.

Details of the apparatus used for the detection of these electrical lines of force are given in the above-mentioned report; however, the general features are shown in Fig. 1.

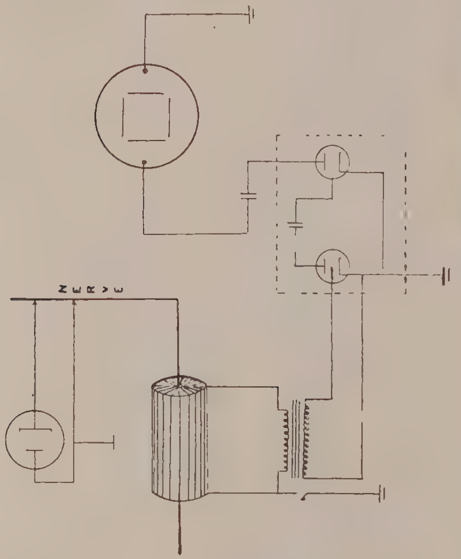

FIG. 1.

That the disturbances indicated by the recording oscillograph were in the nature of induced electrical charges on the detecting coil and not an induced e.m.f. is shown by the fact that they disappeared when the primary coil of the matching transformer was grounded. (*Cf.* Fig. 1.)

The frog's sciatic was used. The results are indicated in Fig. 2. A gives the stimulus artifact (*ca.* 30 m V); B shows the result obtained with an intact nerve, and reveals the 2 peaks; C is the result obtained when the detector is located on the boundary of the intact and crushed portion.

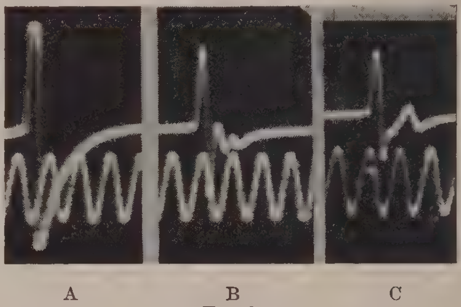

FIG. 2.
A. Stimulus artifact obtained with dead nerve.
B. The two peaks obtained with intact nerve.
C. Single peak obtained when detector is located on boundary of crushed and intact portion of the nerve. Time line indicates 500 cycles.

It was possible to duplicate these results when the nerve and the detector were both immersed in tap water, mineral oil, and Ringer's solution. In the case of Ringer's, it was possible to demonstrate increased velocity of the nerve impulse as being of the order of value calculated by Hodgkin[2] by other experimental means.

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[2] Hodgkin, A. L., *J. Physiol.*, 1939, **94**, 510.

14082

Role of Sodium Chloride in Production of Nephrosclerosis by Steroids.

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Previous investigations[1] have shown that desoxycorticosterone acetate (D.C.A.) causes nephrosclerosis and generalized tissue edema in the chick. The condition is usually accompanied by cardiovascular changes similar to those seen in human hypertensive heart disease. In the present communication we should like to discuss a series of experiments indicating that high doses of NaCl added to the drinking water of chicks may in themselves suffice to elicit all these changes. Such salt treatment causes death due to the development of a condition similar to that of the naturally-occurring Bright's disease[2] or "pullet disease"[3] of the fowl. On the other hand, D.C.A. elicits these changes even in birds kept on a normal salt intake and potentiates the effect of sub-threshold doses of NaCl.

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[1] Selye, Hans, *Canad. Med. Assn. J.*, 1942, **47**, 515.

In the first experimental series 6 male and 6 female 2-day-old single-combed White Leghorn chicks weighing 35-38 g (average 35 g) were given 2% NaCl solution instead of drinking water. All of these birds died with generalized tissue edema and cardiac dilatation within 3 days. The experiment was then repeated on 6 male and 6 female 3-week-old Barred Rock chicks which received 0.9% NaCl instead of drinking water. Eight of these died within 20 days and the remaining 4 were killed at the end of this time. All 12 animals showed generalized tissue edema, fluid accumulations in the serous cavities and cardiac dilatation. Their kidneys were enlarged and histologically characterized by glomerular sclerosis, tubular hypertrophy and numerous casts. This is essentially the same picture as that previously obtained with D.C.A. in chicks kept on a normal salt intake.[1]

Additional experiments were then performed in order to establish whether sub-threshold doses of NaCl—which in themselves are not toxic—could sensitize the bird to the above-mentioned actions of D.C.A. We felt that if this were so we would not only gain further information concerning the pathogenesis of these lesions, but would also acquire a highly sensitive test object, suitable for the estimation of nephrotoxic potency in steroids other than D.C.A. One hundred seventy seven-day old, single-combed White Leghorn pullets were subdivided into 17 groups of 10 birds each. The average initial body weight in each group was 44 g (range 38-52 g). At such an early age it is difficult to tell the sex of live pullets but autopsy at the end of the experiment revealed that the number of males and females was approximately the same in the various groups. All the steroids assayed were administered subcutaneously in the form of finely ground crystals suspended in water. Two mg in 0.2 cc of distilled water were given twice daily. This injection treatment was combined with the administration of an 0.2% NaCl solution instead of ordinary drinking water. This weak solution does not cause any nephrosclerosis when given by itself as shown by previous experiments and by the group receiving it in combination with inactive steroids (*e.g.* cholesterol). However, we anticipated that it would sensitize the birds to nephrotoxic steroids. In order to show this sensitization, one group of D.C.A. treated birds received the above NaCl solution while another received tap water for comparison. It has been claimed that potassium salts antagonize many actions of sodium and that certain D.C.A. overdosage symptoms are counteracted by potassium ions.[4,5] Hence in one group we administered the above NaCl solution with KCl added in an equimolecular concentration. A third group received only the KCl solution of this same concentration (0.25%). All animals were killed 10 days after initiation of the treatment and the prominent findings are summarized in Table I. The common names of the various steroids are given in italic letters and the systematic chemical names are likewise mentioned as well as the melting point of the specimen which we used. This will help to identify the compounds in a definite manner and to indicate the degree of purity of our preparations.

Perusal of the table indicates that an NaCl solution which in itself is not toxic to the chick becomes toxic if given in conjunction with comparatively small doses of D.C.A. The toxicity of this corticoid—as evidenced by the degree of edema and the number of deaths caused—is about twice as marked in

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[2] Weaver, C. H., *Proc. 14th Ann. Conf. Lab. Workers in Pullorum Disease,* Ottawa, Canada, May, 1941.

[3] Jungherr, Erwin, and Levine, Jacob M., *Am. J. Vet. Res.*, 1941, **2**, 261.

[4] Pudenz, Robert H., McIntosh, John F., and McEachern, Donald, *J. Am. Med. Assn.*, 1938, **111**, 2253.

[5] Kuhlmann, Daniel, Ragan, C., Ferrebee, J. W., Atchley, Dana W., and Loeb, Robert F., *Science*, 1939, **90**, 496.

TABLE I.

Steroid treatment	M.P.°C	Salt treatment	No. of deaths before 10th day	Degree of edema
Desoxycorticosterone acetate 17-ethyl-Δ^4-androstene-3,20-dione-21-ol acetate	152	tap water	4	++
Desoxycorticosterone acetate	152	NaCl	8	++++
Desoxycorticosterone acetate	152	KCl	3	++
Desoxycorticosterone acetate	152	NaCl + KCl	6	++++
Progesterone 17-ethyl-Δ^4-androstene-3,20-dione	128	NaCl	4	++
Pregnenolone 17-ethyl-androstene-3(β)-ol-20-one	186	”	0	0
Pregnanedione 17-ethyl-etiocholane-3,20-dione	121	”	0	0
Δ^{16}-*pregnenedione* 17-ethyl-Δ^{16}-etiocholane-3,20-dione	190	”	0	0
Ethinyl testosterone 17-ethinyl-Δ^4-androstene-3-one-17(α)-ol	265-268	”	0	0
Testosterone Δ^4-androstene-3-one-17(α)-ol	154	”	0	0
Androstenediol Δ^5-androstene-3(β),17(α)-diol	182-183	”	0	0
Dehydro-iso-androsterone Δ^5-androstene-3(β)-ol-17-one	137	”	0	0
Androstenedione Δ^4-androstene-3-17-dione	169-171	”	0	0
Estradiol $\Delta^{1,3,5}$-estratriene-3,17(α)-diol	176-177	”	0	0
*Adrenal cortical extract**	—	”	0	0
Nor-cholestenolone 17-*iso*-heptyl-Δ^5-androstene-3(β)-ol-25-one	127-128	”	0	0
Cholesterol 17-*iso*-octyl-Δ^5-androstene-3(β)-ol	149	”	0	0

*Prepared by Professor E. C. Kendall. 1 cc represents 75 g of fresh adrenal glands and contains approximately 40 units as defined by Selye and Schenker.[6] 0.25 cc were given twice daily subcutaneously.

chicks receiving the NaCl solution than in those given tap water. Equimolecular doses of KCl do not appear to exhibit this potentiating action of NaCl, nor can they inhibit the effect of the latter. Progesterone, which like D.C.A. exhibits definite corticoid actions, is the only other steroid among those investigated, which is toxic to NaCl sensitized chicks. It is of interest in this connection that progesterone also shares with D.C.A. the diuretic effect of the latter.[7] Further experiments will have to show whether other corticoids, especially the carbohydrate active ones oxygenated at C_{11}, exhibit this effect. It is noteworthy, however, that the adrenal extract, which is rich in such corticoids, caused no edema at least in the doses used.

The histologically detectable renal changes were not as pronounced as in the more chronic experiments mentioned above,[1] however, they were qualitatively of the same type both in the D.C.A. and in the progesterone series (see Fig. 1-3). The convoluted tubules showed signs of hypertrophy and sometimes cloudy swelling with desquamation. Many of them contained hyaline casts. The glomeruli were enlarged and in many cases the cells of the parietal lamina of Bowman's capsule were unusually high. There also was some hyalinization of glomerular capillaries and proliferation of epithelioid cells in the region of the glomerular capsule. No histological changes were detectable in the kidneys of the chicks receiving compounds other than D.C.A. or progesterone.

Although the chick is by far the most sensitive test animal for this particular effect of the steroids it should be mentioned that essen-

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[6] Selye, Hans, and Schenker, Victor, PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 518.

[7] Selye, Hans, and Bassett, Lucy, PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 502.

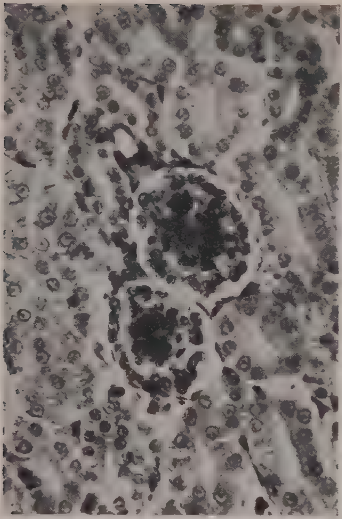

Fig. 1.
Normal kidney of chick receiving NaCl and cholesterol.

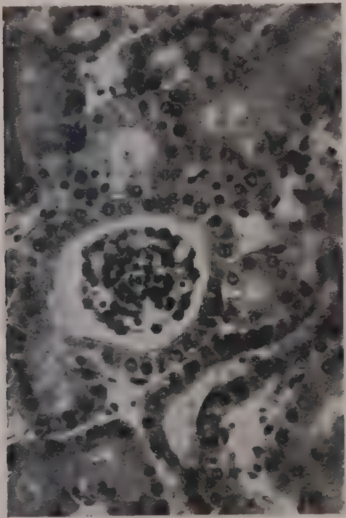

Fig. 2.
Kidney of chick receiving NaCl and D.C.A. Note hypertrophy of convoluted tubules and of parietal lamina of Bowman's capsule.

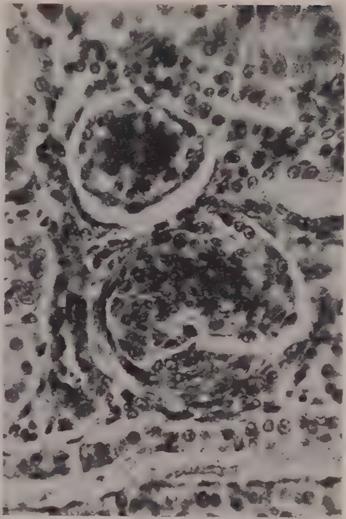

Fig. 3.
Kidney of chick receiving NaCl and progesterone. Note "epithelioid crescent" in one of the renal corpuscles.

tially similar renal lesions have also been produced in the rat, dog and monkey.[8] Hence it is not impossible that disturbances in water metabolism and certain types of nephrosclerosis may occur even in man as a result of excessive production of D.C.A., progesterone or other pharmacologically related steroids.

Conclusions. Administration of concentrated NaCl solutions causes generalized tissue edema and nephrosclerosis in young chicks. Desoxycorticosterone acetate (D.C.A.) exerts a similar effect even on a normal salt intake and sensitizes chicks to the action of subthreshold concentrations of NaCl. Among a number of steroid preparations tested for this effect only progesterone was found to possess this activity and even this compound was not quite as active as D.C.A.

The expenses of this investigation were defrayed through a grant received from the DesBergers-Bismol Laboratories of Montreal, Canada. We are greatly indebted to Dr. Erwin Schwenk of the Schering Corporation of Bloomfield, N.J., for all steroid compounds except the pregnanedione which was supplied by Professor R. D. H. Heard and the Δ^{16}-pregnenedione donated by Dr. O. Kamm of Parke, Davis and Company. Our thanks are also due to Dr. S. S. Munro of the Canadian Department of Agriculture who furnished the chicks for these experiments.

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[8] Selye, Hans, and Hall, C. E., *Arch. Path.*, in press.

14083

Magnetic Susceptibility of Beef Tendon.

K. S. Lion. (Introduced by F. O. Schmitt.)

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That tendon fibers show marked mechanical and optical anisotropy has long been known. In addition to these properties, the magnetic anisotropy of tendon has been studied. Measuring the magnetic susceptibility of beef tendon parallel and perpendicular to the fiber axis, Wöhlisch[1] concluded that the tendon shows positive paramagnetic properties, *i.e.*, the susceptibility is greater parallel than perpendicular to the fiber axis. His method was only qualitative, consisting of the observation of the rotation of the fiber freely suspended between the poles of a magnet, hence no numerical value for the susceptibility could be given.

While some degree of magnetic anisotropy is to be expected in such fibers it seems probable that, like most organic substances, they would be diamagnetic rather than paramagnetic. Gelatin, which is closely related to collagen, was found to be diamagnetic both in the dry form (Ambronn, quoted by Wöhlisch) and in approximately 1% solutions (Fallot[2]). The susceptibilities obtained by the latter investigator ranged between -0.76×10^{-6} and -0.91×10^{-6}.

In considering the cause of this apparent discrepancy it seemed possible that Wöhlisch's result may have been due to contamination of the material with traces of iron derived from the instruments with which the tendon was cut.

The magnetic susceptibility of iron at low field strength is of the order of 10^3. One gram of diamagnetic substance of the susceptibility of 10^{-6} will appear to be paramagnetic, therefore, if the content of iron is higher than 10^{-9} g.

If a sample consisting of such a mixture of a ferromagnetic and a (presumably) diamagnetic substance is brought into a nonhomogeneous magnetic field, two forces will act on the sample: one, resulting from the diamagnetic component, in one direction (Curve *A*, Fig. 1); the other, resulting from the ferromagnetic component, in the other direction (Curve *B*). However, Curve *B* shows saturation with increased field strength. A mixture of a diamagnetic and a ferromagnetic substance is characterized by Curve *C*. If, on the other hand, the body is composed of a ferromagnetic and a paramagnetic substance, the force acting on it will correspond to the Curve *E*, resulting from a super-position of Curves *B* and *D*.

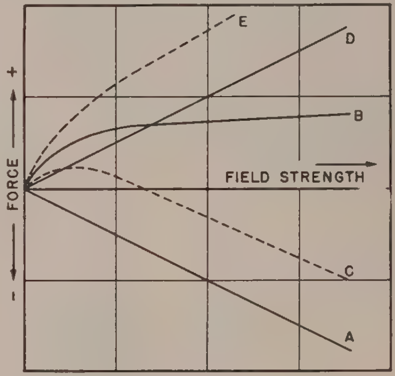

Fig. 1.

Forces acting on a body in a nonhomogeneous magnetic field. Body *A*—Diamagnetic, *B*—Ferromagnetic, *C*—Mixture of *A* and *B*, *D*—Paramagnetic, *E*—Mixture of *B* and *D*.

To test the correctness of this interpretation of Wöhlisch's result the following experiment was performed. The dried tendon fiber was suspended by a silk fiber from one arm of an analytical balance into the field of a large coreless magnet.* The force acting on the sample is:

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[1] Wöhlisch, *Verh. d. physik.-med. Ges. Würzburg*, 1926, **51**, 63; *Z. f. Biologie*, 1926, **84**, 598.

[2] Fallot, *Comptes Rendues*, 1929, **188**, 1498.

* For the use of this magnet we are indebted to Prof. A. R. Kaufman of the Metallurgy Department of M.I.T.

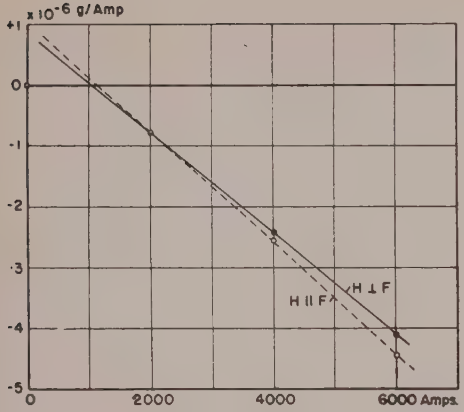

Fig. 2.
Explanation see text.

$$f = m \cdot \chi \cdot H \cdot \frac{\delta H}{\delta x}$$

where m is the mass, χ the susceptibility, H the strength of the magnetic field, and $\delta H/\delta x$ the inhomogeneity of the field in a vertical direction in the region occupied by the tendon. H could be varied from 0-36,000 Gauss by varying the current from 0-6,000 Amps.

Fig. 2 illustrates the results obtained. All measured points at field strengths above 6,000 Gauss show a deflection to the negative side, indicating that beef tendon is *diamagnetic*. The numerical value[†] of the susceptibility was found to be $-0.80_7 \cdot 10^{-6}$ parallel to the fiber axis, and $-0.85 \cdot 10^{-6}$ perpendicular to the fiber axis. The fiber is therefore *negatively diamagnetic*. The presence of traces of iron is assumed from the fact that the line connecting the measured points does not pass through the origin. It seems probable that Wöhlisch's anomalous result was due to a similar cause.

[†] Unfortunately only 2 experiments could be performed before the magnet was required for other purposes. The data are, therefore, not sufficient to establish accurately the absolute value of the susceptibility. However, the purpose of the work was to explain the anomalous results of Wöhlisch rather than to determine the numerical value of the susceptibility which, in itself, is not particularly significant.

14084

Response of Human Pregnant Uterus to Pitocin Tannate in Oil.*

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The posterior pituitary hormone was fractionated by Kamm and his associates[1] into the pressor-antidiuretic fraction (Pitressin) and the oxytocic fraction (Pitocin). Two years ago a preparation of Pitressin tannate in oil (a tannic acid precipitate of the Pitressin fraction resuspended in peanut oil) was utilized in the treatment of diabetes insipidus[2] because of its prolonged action. At this time a similar preparation of the Pitocin fraction was prepared[†] for clinical trial in the induction of labor and for uterine inertia. It was hoped that a more slowly absorbed oxytocic would produce a more physiologic response than the rapidly acting aqueous posterior pituitary hormone.

Methods. Injections of Pitocin tannate in oil were given to 22 women for the induction of labor at term and to 10 women whose labors were complicated by primary uterine inertia. In many instances a graphic record of the response was obtained by means of the Lorand

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* Aided by a grant from the John and Mary Markle Foundation.

[1] Kamm, O., Aldrich, T. B., Grote, I. W., Rowe, L. W., and Bugbee, E. P., *J. Am. Chem. Soc.*, 1928, **50**, 573.

[2] Greene, J. A., and January, L. E., *J. A. M. A.*, 1940, **115**, 1183.

[†] The Pitocin tannate in oil was prepared and donated by Doctors Oliver Kamm and E. A. Sharp of Parke, Davis and Company.

tocograph.[3] The responses were classified as clonic, incomplete tetanic or tetanic according to the descriptions of Murphy.[4] An attempt was made to study (a) the latent period between the time of injection and onset of response, (b) the dosage ratio between Pitocin tannate in oil and Pitocin, (c) the comparative duration of action of the two preparations, and (d) the qualitative nature of the uterine response. In each case, the minimal effective dose of Pitocin (10 units per cc) was determined before administering Pitocin tannate in oil (8 units per cc). Pitocin was diluted 10 times and the initial dose of 0.25 unit was doubled every 20 to 30 minutes until the first painful uterine contractions were noted. After this dosage was determined, twice the amount of Pitocin tannate in oil was injected intramuscularly in the lower third of the upper arm and repeated every 20 minutes until the same uterine response was noted. The depot was placed in the lower portion of the arm so that a blood pressure cuff above could stop further absorption in the event of stormy or tetanic responses. Such a tourniquet was necessary and proved valuable in one case of uterine inertia in which too large an amount of the hormone in oil was administered. The cuff was intermittently inflated above systolic pressure for periods of 10 minutes until the tetanic types of responses decreased.

Results. The latent period between the time of injection of Pitocin tannate in oil and the onset of response was usually 8 to 10 minutes (extremes of 5 to 16 minutes), or about twice as long as the latent period for Pitocin.

The ratio of minimal effective dose of Pitocin tannate in oil to Pitocin varied from 2 to 6, usually 4 times the number of units. In other words, 0.1 cc (1 unit) of Pitocin was equivalent to about 0.5 cc (4 units) of the preparation in oil. Variations in the uterine threshold from time to time in the same woman prevents a more exact determination of the dosage ratio.

The duration of action is difficult to determine in most instances because spontaneous labor may supervene. An estimate of the duration is therefore based upon 10 cases whose labor pains did not continue, and the cessation of painful uterine contractions was assumed to coincide with the cessation of pharmacologic response. The shortest response was 28 minutes and the longest 150 minutes, the duration of action in 6 of the 10 cases being between 45 and 90 minutes, or 3 to 4 times as long as with ordinary Pitocin. Tracings of 2 tocograph records on the same woman, made one hour apart during the induction of labor at term, illustrate the differences in latent period and duration of response resulting from the two preparations. In both instances an incomplete tetanic response resulted. (See Fig. 1.)

Dieckmann and Kharasch[5] recently described similar experiences with a solution of posterior pituitary sulfonate, prepared by precipitating the hormone with anthra-quinone 2·6 disulphonic acid and resuspending in acid solution. Free posterior pituitary hormone is liberated slowly when it becomes alkaline, as with intramuscular injection. They concluded that the "Pit Sulphonate" differed from the usual solution in that there was little if any likelihood of tetanic contractions even though a large dose is given and that the preparation may be used with comparative safety to both mother and fetus.

It is difficult to see how modifying the rate of absorption of an oxytocic can eliminate the dangers resulting from overdosage. With both Pitocin tannate in oil and ordinary Pitocin the same frequency of clonic, incomplete and complete tetanic responses were encountered with equivalent doses. After the limited experiences described above the writer hesitates to use the suspension in oil in preference to Pitocin for uterine inertia because any untoward effects from overdosage are unduly prolonged. The most physiologic responses in severe primary uterine inertia have actually been obtained by giving an intravenous infusion of normal saline containing either 10 or 20 units of Pitocin to the liter. By constant observation and controlling the number of

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[3] Lorand, S., *Monatschr. f. Geburtsh. u. Gynäk.*, 1936, **103**, 137.

[4] Murphy, D. P., *Am. J. Obst. and Gynec.*, 1941, **41**, 274.

[5] Dieckmann, W. J., and Kharasch, M. S., *Am. J. Obst. and Gynec.*, 1942, **44**, 820.

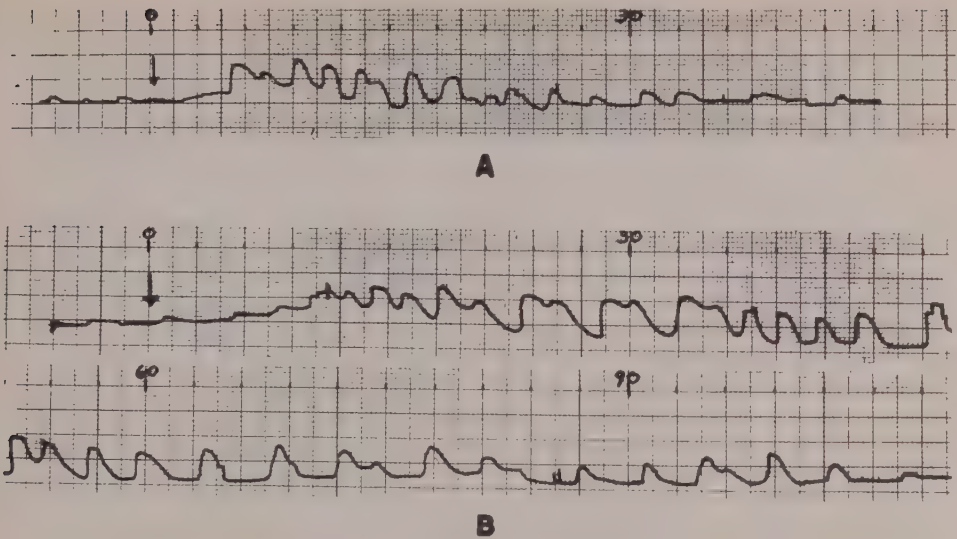

FIG. 1.

Tracings of tocograph records made on the same patient at term, not in labor, showing (A) the uterine response to 2 units of Pitocin and (B) the response one hour later to 7 units of Pitocin tannate in oil. Time in 3-minute intervals.

drops per minute, the obstetrician may control the character and amplitude of contractions quite adequately. The latter technic deserves further trial in selected cases of marked uterine inertia.

Conclusions. Pitocin tannate in oil, in comparison with Pitocin, requires 3 to 4 times the dosage to elicit a comparable response; the latent period after injection is twice as long; the duration of action 3 to 4 times as long. The preparation may be used when a longer acting oxytocic is desirable but possesses the same inherent dangers of ordinary posterior pituitary hormone.

14085 P

Differentiation Between *Brucella melitensis* and *Brucella abortus* by Precipitin Reaction.

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The precipitin reaction has been used in an attempt to develop a simple and rapid method by which strains of *Brucella melitensis* can be differentiated from those of *Brucella abortus.* Strains utilized for the immunization of rabbits were *B. melitensis* 428 and *B. abortus* 456 obtained from the National Institute of Health at Washington, and *B. suis* 1529, isolated by Huddleson in 1931 from the supramammary gland of a hog.

The organisms were grown for 48 hours on freshly prepared tryptose agar slants and the growth suspended in 0.9% saline, the opacity adjusted to match barium sulphate standard No. 3. One-tenth of a cc of this living suspension was then injected into the ear vein of rabbits every other day until 4 injections had been given. On successive days following the last injection, trial bleedings were made from the ear vein and precipitin reactions carried out with formamide extracts of homologous organisms. If after 10 days precipitins were not demonstrable, another series of injections was started, followed by daily bleedings. Such a procedure was continued until precipitins appeared in the blood, their presence being indicated by a definite ring at the interphase between the serum and the extract and appearing in about 10 to 15 minutes. Immediately thereafter, the animal was bled and the blood collected into sterile paraffined test tubes; the serum was then used for the tests. Emphasis must be laid on the fact that the animal has to be bled as soon as the presence of precipitin in the blood is detected.

The preparation of the extract followed Fuller's[1] method for the extraction of polysaccharides from hemolytic streptococci. In this case, however, the organisms were grown for 48 hours on tryptose phosphate agar slants and the amount of growth, that could be scraped off at one time with a loop 1.5 mm in diameter, used for the extraction.

With a fine capillary pipette, 2 or 3 drops of the immune serum were placed in a shell vial 25 x 8 mm and carefully overlaid with an equal volume of the extract to be tested. The tube, set aside at room temperature, was watched continuously during half an hour for the appearance of a precipitate at the interphase. Only the limited number of strains in our collection were tested.*

Each extract was tested against *mellitensis, suis,* and *abortus* immune sera. All of the *abortus* and *suis* extracts promptly produced a definite ring with both *abortus* and *suis* sera, yet they did not produce a precipitate with the *melitensis* serum even in thirty minutes.

The *melitensis* extracts produced a precipitate readily with the *melitensis* serum. Three of these failed to precipitate in 30 minutes, when tested against *abortus* and *suis* sera, but one (*B. melitensis* 662) did. When originally tested and, when the usual amount of growth for the preparation of the extract was utilized, strain 662 gave a faint delayed reaction with *melitensis, suis* and *abortus* sera. However, when a larger amount of growth was used, a ring was readily obtained with the three antisera. Such results suggested the presence in this particular strain of a smaller amount of the specific polysaccharide than is usually present in the typical *melitensis* cell. It must be remembered that intermediate strains, containing about the same proportion of *abortus* and *melitensis* polysaccharides, may exist, hence for immunization it is important to use the proper strain, typical in all respects. It is obvious that if a strain like 662 is used, a precipitating serum obtained will be useless in the differentiation of organisms of the Brucella group.

Dried and finely powdered organisms, kept in a desiccator for 2 years, were found suitable for the preparation of extracts and for the immunization of animals. Extracts prepared from six-months-old cultures on tryptose agar also produced good precipitates with their corresponding antisera.

The method herein described is simple and rapid and the results are clear-cut. It is useful in the study of the classification and of the antigenic structure of the Brucella group of organisms.

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[1] Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 1930.

* *B. abortus* strain 456, obtained from the National Institute of Health in Washington, and No. 1265 isolated by Huddleson from an aborted bovine fetus in 1940. *B. suis* strains 1670 and 1529, isolated by Huddleson from human blood in 1939 and from the supramammary gland of a hog in 1931, respectively, and a *suis* strain obtained from the National Institute of Health. *B. melitensis* strains 2456, 2466, and 662, isolated by Huddleson from human blood in 1939, 1940, and 1930, respectively, and strain 428 obtained from the National Institute of Health of Washington.

14086 P

Preparation of Pituitary Adrenotropic Hormone.*

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This is a preliminary description of a relatively simple procedure devised for the isolation of purified and highly active preparations of adrenotropic hormone from whole hog pituitary glands. The extraction and fractionation procedures consist of the following steps: (1) Extraction and isolation of a fraction designated as crude prolactin by the procedure originally developed by Lyons[1] and adopted by White, Bonsnes and Long[2] as the first step for the subsequent preparation of crystalline prolactin. This "crude prolactin" contains adrenotropic hormone activity. (2) Solution of the crude prolactin preparation in 2% concentration at pH 9.0 and removal of precipitates which form by careful adjustment of pH to 8.0, 6.6 and 5.4. (3) Supernatant obtained in Step 2, after removal of pH 5.4 insoluble material (prolactin), is freed from traces of prolactin by the addition of 7 ml of saturated $(NH_4)_2SO_4$ to each 100 ml of solution. The precipitate is separated and discarded. (4) Acetone is added to the supernatant to a concentration of 80%. The adrenotropic fraction precipitates. (5) This precipitate is dissolved in water (volume used being two-thirds of that employed in Step 2) and the solution mixed with one-half its volume of concentrated NH_4OH. The ammoniacal solution is allowed to stand at room temperature for 7 hours. (6) Acetone is then added to above solution to a concentration of 90%. (7) The precipitate is dissolved in one-third volume of water used in Step 2, and dialyzed to remove salt. (8) The adrenotropic fraction is precipitated by lowering of pH to 4.7. The precipitate may be dried by washing 3 times with acetone at the centrifuge and finally drying *in vacuo* over concentrated sulfuric acid. The yield of dry, purified adrenotropic fraction has been of the order of 500 mg from each 1000 g of whole hog pituitary glands.

It may be noted that the yield of the crude prolactin fraction is strikingly higher, approximately 3 to 4 times, from hog pituitary glands than from a similar weight of beef pituitary tissue.

The adrenotropic preparations have been assayed by two methods: (1) restitution of atrophied adrenals in 45-day-old, hypophysectomized, male rats, 10 days post-operative; and (2) maintenance of adrenal size in 45-day-old, male hypophysectomized rats. Using the first method of assay, a total of 5 mg of adrenotropic hormone preparation, given 3 times daily for 3 days, resulted in restoration of the adrenal size to that found in unoperated control animals of the same age. The maintenance method of assay showed that 0.05 mg of adrenotropic hormone preparation, given daily for 14 days beginning at the first day postoperative, was adequate for maintaining normal adrenal size. The adrenotropic preparations thus have a potency of the order of that of the preparation recently described by Li, Simpson and Evans.[3] The assay data are summarized in Table I.

From preliminary data available at the present time, the purified adrenotropic hormone preparations give no definite evidence of

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* This investigation has been aided by grants from the Fluid Research Fund, Yale University School of Medicine, The Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association, and The Committee on Research in Endocrinology, National Research Council.

[1] Lyons, W. R., PROC. SOC. EXP. BIOL. AND MED., 1936-37, **35**, 645; in *Cold Spring Harbor Symposia on Quantitative Biology*, Cold Spring Harbor, 1937, **5**, 198.

[2] White, A., Bonsnes, R. W., and Long, C. N. H., *J. Biol. Chem.*, 1942, **143**, 447.

[3] Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, 1942, **96**, 450.

TABLE I.
Assay of Adrenotropic Hormone Preparation in 45-day-old Hypophysectomized Male Rats.

Type of rat	No. of animals	Avg change in body wt,* g	Avg paired adrenal wt, mg	Avg paired testes wt, mg
Intact, normal	9		24.2 (19.3-30.0)†	2,266 (1,890-2,660)†
Hypophysectomized, uninj. control	13	—15	12.1 (9.0-15.8)	480 (234-700)
Hypophysectomized, inj. 3 times daily 3 days, total dose 5 mg A.C.T.,‡ beginning 10 days postoperative	9	—15	20.0 (16.5-29.9)	676 (390-1,130)
Hypophysectomized, inj. daily 14 days with 0.1 mg A.C.T. per day, beginning first day postoperative	12	—12	23.5 (20.0-26.3)	372 (154-573)
Hypophysectomized, inj. daily 14 days with 0.05 mg A.C.T. per day, beginning first day postoperative	9	— 7	20.8 (17.5-26.5)	285 (119-636)

*Between day of operation and time of autopsy.
†Figures in parentheses indicate range of values observed.
‡A.C.T. = adrenotropic hormone preparation.

the presence of the following types of pituitary activities: lactogenic, growth-promoting, gonadotropic, or thyrotropic. Assays for posterior pituitary principles indicate that one milligram of the adrenotropic preparation has a pressor and antidiuretic activity equivalent to approximately 0.6 unit of Pitressin (Parke-Davis).[4] The purified adrenotropic hormone fraction is homogeneous in the Tiselius apparatus, at four different hydrogen ion concentrations, and in the ultracentrifuge.

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[4] Sayers, G., Sayers, M. A., White, A., and Long, C. N. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 200.

14087 P

Effect of Pituitary Adrenotropic Hormone on Cholesterol Content of Rat Adrenal Glands.*

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In the course of studies of the physiological effects of adrenotropic hormone, striking decreases in adrenal cholesterol content were found to occur in normal 24-day-old male rats within 3 hours following administration of 2 mg of the hormone preparation described in the preceding paper.[1] Subsequently, the adrenal cholesterol concentration returns to normal within 24 hours after hormone injection. Continued injection of the adrenotropic hormone for 3 days raises the cholesterol content of the adrenals to values above those observed in adrenals of normal, uninjected rats.

The rats on which adrenal cholesterol data

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* This investigation has been aided by grants from the Fluid Research Fund, Yale University School of Medicine, The Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association, The Committee on Research in Endocrinology, National Research Council, and The Josiah Macy, Jr., Foundation.

[1] Sayers, G., White, A., and Long, C. N. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 199.

TABLE I.

Adrenal Cholesterol Values of Normal and Adrenotropic Hormone Treated Male Rats, Each 24 Days Old.

		Cholesterol concentration in adrenal glands			
Type of rat[*]	No. of animals	Mg/100 mg fresh adrenal tissue	P value	Mg/100 g body wt	P value
Control, injected with water	22	3.5 ± 0.18[†]		1.12 ± 0.06	
3 hr after inj. of 2 mg A.C.T.[‡]	16	1.6 ± 0.08	<0.01[§]	0.57 ± 0.03	<0.01
6 hr after inj. of 2 mg A.C.T.	4	2.0 ± 0.12	<0.01	0.73 ± 0.06	0.02
9 hr after inj. of 2 mg A.C.T.	4	2.3 ± 0.18	0.02	0.83 ± 0.09	0.05
24 hr after inj. of 2 mg A.C.T.	5	3.6 ± 0.07	0.8	1.37 ± 0.08	0.05
Inj. thrice daily 3 days, total 2 mg A.C.T.	4	4.4 ± 0.11	0.05	1.68 ± 0.11	<0.01
Inj. thrice daily 3 days, total 5 mg A.C.T.	5	5.0 ± 0.4	<0.01	2.52 ± 0.25	<0.01
3 hr after inj. 1.0 pressor unit pitressin (Parke-Davis)	9	3.0 ± 0.13	0.1	0.92 ± 0.08	0.05

[*] All animals received food and water *ad libitum.*

[†] Means and standard errors. $\text{Standard error} = \sqrt{\dfrac{\text{sum of squares of deviations from mean,}}{n\ (n-1)}}$

where n = number of observations.

[‡] A.C.T. = adrenotropic hormone preparation.

[§] P values as compared to control groups.

are here reported were obtained from two sources, from the Biochemical Laboratory of the Connecticut Agricultural Experiment Station, through the courtesy of Dr. Rebecca Hubbell, and from The Breeding and Laboratory Institute, Brooklyn. Twenty-four-day-old male animals were used. Only healthy, growing rats are suitable for the studies of adrenal cholesterol changes inasmuch as it has been found that inanition or infection will markedly deplete the adrenals of cholesterol. Similar observations on the effect of infection on the adrenal cholesterol level have been recently reported by Sperry and Stoyanoff.[2] Injections were made intraperitoneally, generally in a volume of 0.25 ml for each injection. At the appropriate time, the rats were killed with illuminating gas, the adrenals carefully dissected out and weighed. Total cholesterol determinations were conducted on either single or paired adrenal glands of each rat by the method described by Sperry.[3]

In view of the fact that the purified adrenotropic hormone preparations exhibited slight pressor (cat)[†] and antidiuretic (rat) activity,[4] the action of posterior pituitary pressor substance (Parke-Davis) on adrenal cholesterol levels was examined. One milligram of purified adrenotropic hormone was found to contain an amount of pressor and antidiuretic activity equivalent to 0.6 unit of Parke-Davis Pitressin.

The cholesterol data which have been obtained are presented in Table I. The standard errors of each series are also included in the table, together with the P values, which have been calculated by the method of Fisher[5] for small series in order to determine which differences among the several groups of experimental animals are significant.

The data indicate a definite lowering of the adrenal cholesterol level 3 hours after injection of adrenotropic hormone. Subsequently, the adrenal cholesterol concentration rises and returns to normal within 24 hours following hormone administration. Moreover, the repeated injection of the hormone over a period of 3 days raises the adrenal cholesterol concentration above that of control animals. This is particularly striking with a total dose of 5 mg of the adrenotropic preparation. It is to be noted that the increases in cholesterol concentration are greater when calculated on the basis of animal body weight. This is due to the fact that adrenal hypertrophy is occurring under the influence of the hormone, and cholesterol is being deposited in the new adrenal tissue in a concentration at least as great as that present in the adrenal gland before stimulation.

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[2] Sperry, W. M., and Stoyanoff, V. A., *J. Nutrition*, 1938, **9**, 131.

[3] Sperry, W. M., *Am. J. Clin. Path.*, Tech. Suppl., 1938, **2**, 91.

[†] Grateful acknowledgment is made to Dr. L. S. Goodman of the Department of Pharmacology for the pressor assay in the cat.

[4] Gilman, A., and Goodman, L. S., *J. Physiol.*, 1937, **90**, 113.

[5] Fisher, R. A., *Statistical Methods for Research Workers*, 1932, 4th Ed., Oliver and Boyd, London.

The results in Table I suggest that the adrenotropic hormone is concerned with the regulation of metabolic changes in the adrenal gland involving cholesterol. In view of the fact that the active principles of the adrenal cortex are also steroids, it is possible that variations in the cholesterol content of the adrenal also reflect changes in concentration of steroid hormones. This suggestion finds support in evidence of increased function of the adrenal cortex following adrenotropic hormone administration, as measured by liver glycogen changes[6] and in the report[7] that adrenotropic hormone increases the resistance of hypophysectomized and normal rats to cold, starvation and anoxia.

It may be added that the cholesterol concentration of the adrenals of 45-day-old hypophysectomized rats has been found to be elevated in comparison to the cholesterol levels in the adrenals of unoperated animals of the same age. Cholesterol data similar to those which are reported have also been obtained by employing 2 mg of an adrenotropic hormone fraction prepared from whole sheep pituitary glands by a procedure essentially that described by Li, Simpson and Evans.[7]

Summary. Pituitary adrenotropic hormone produces a distinct lowering of the adrenal cholesterol level 3 hours after injection of 2 mg into 24-day-old male rats; repeated injections over a period of 3 days results in an increase in adrenal cholesterol concentration above that of control animals.

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[6] Grattan, J. F., and Jensen, H., *J. Biol. Chem.*, 1940, **135**, 511.

[7] Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, 1942, **96**, 450.

14088 P

Excretion of Keto Acids and Hydroxyphenyl Compounds in Pernicious Anemia.

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It has been found in this laboratory that the urinary excretion values for keto acids of patients with untreated pernicious anemia in relapse show a marked decrease when liver extract is administered by intramuscular injection. Since there are several indications that tyrosine metabolism may be disturbed in pernicious anemia, it was of interest to measure the urinary hydroxyphenyl compounds in this disease in order to determine whether or not their excretion values could be correlated with those of the keto acids. Previous experiments by Becher[1] have shown a rise in blood phenols in pernicious anemia and, in contrast, Volterra[2] observed a low excretion of volatile phenols in the urine in untreated pernicious anemia and a greatly increased excretion of these compounds after liver therapy.

Twenty-four-hour urine samples were collected in acid and analyzed for keto acids by

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[1] Becher, E., Litzner, S., and Täglich, W., *Z. klin. Med.*, 1926, **104**, 195.

[2] Volterra, A., *Schweiz. med. Wochschn.*, 1939, **69**, 627.

TABLE I.
Urinary Excretion Daily Values for Keto Acids and Hydroxyphenyl Compounds in Pernicious Anemia.

	Keto acids expressed as pyruvic acid in mg per 24 hr.				Hydroxyphenyl compounds expressed as tyrosine in mg per 24 hr			
	Relapse		Remission		Relapse		Remission	
Patient	Range	Avg	Range	Avg	Range	Avg	Range	Avg
W.H.	228-177	202	107-94	99	741-444	584	292-242	278
H.U.	158-107	135	63-56	60	799-427	513	290-270	280
S.H.	153-103	138	97-68	76	428-308	400	260-201	219
C.O.	135-124	127	93-74	82	682-362	442	287-240	256

the method of Penrose and Quastel[3] and for hydroxyphenyl compounds by the Medes modification of the method of Folin and Ciocalteu.[4] Analyses were made on 4 patients with pernicious anemia before and after liver therapy and, for comparison, on 10 patients receiving the same hospital diet who were apparently healthy except for traumatic injuries.

The analyses on the comparison group gave values for urinary keto acids expressed as pyruvic acid, ranging from 73 to 100 mg per 24 hours with an average of 82 mg. For urinary hydroxyphenyl compounds expressed as tyrosine, the values range from 150 to 230 mg per 24 hours with an average of 195 mg.

The data for the pernicious anemia cases are shown in Table I. At least 4 twenty-four-hour collection specimens were employed in determining the average and range values.

These results indicate that patients with untreated pernicious anemia have an increased excretion of keto acids and hydroxyphenyl compounds, but that after therapy the excretion levels are similar to those of healthy individuals. In every instance, the hydroxyphenyl compounds parallel the keto acids in their excretion values. This would suggest that the keto acid which is excreted in increased amounts in untreated pernicious anemia is an hydroxyphenyl compound. However, since, in both cases, the methods used determine a group of chemically related compounds, this finding does not preclude the possibility that other keto acids or other compounds with hydroxyphenyl groups are involved. Experiments designed to clarify these relations are in progress. The fall in excretion levels of both the keto and hydroxyphenyl compounds in pernicious anemia after institution of therapy is simultaneous with the beginning of the reticulocyte response and precedes any other change in the cellular components of the blood.

These findings point the way to a more detailed consideration of the metabolism of keto and hydroxyphenyl compounds in pernicious anemia.

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[3] Penrose, L., and Quastel, J. H., *Biochem. J.*, 1937, **31**, 266.

[4] Medes, G., *Biochem. J.*, 1917, **26**, 917.

14089

Cultivation of the West Nile Virus in the Developing Chick Embryo.

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The developing chick embryo has been employed with considerable success for the cultivation of a number of neurotropic viruses.[1] The present investigation deals with the cultivation in the embryonated egg of another neurotropic virus, namely, the West Nile agent, recently isolated by Smithburn and his coworkers.[2] Mice, Rhesus monkeys[2] and Syrian hamsters[3] are susceptible to this agent but guinea pigs and rabbits are resistant.[2]

Conventional methods were used for the inoculation of developing embryos. The first eggs were infected with a 10% suspension of brain from a hamster moribund with the West Nile disease: this material, when injected intracerebrally in a dilution of 10^{-6}, killed mice. The infected embryos became sluggish within a few days after injection and at this time material was removed aseptically for passage. Various portions of the passage embryos, as well as their membranes and fluids were studied for amount of virus present. Tissues were ground in a mortar and 10% suspensions were prepared with buffered saline containing 1% normal guinea pig serum.

The West Nile virus multiplies well in embryonated egg inoculated by various routes, and can be readily maintained by serial passage of several types of tissue (Table I). Embryos became sluggish 2 to 4 days after inoculation and death usually occurred within the ensuing 24 hours; the disease progressed more rapidly in the eggs inoculated with large quantities of virus. Death occurred with such regularity following inoculation of virus on the chorioallantoic membrane of 10-day embryos or into the yolk sac of 5-day embryos that virus titrations could be satisfactorily carried out by this technic. Comparative titrations done simultaneously in eggs and in mice with dilutions of the 5th passage chorioallantoic membrane showed that eggs were slightly more susceptible than were mice, *i.e.*, a 10^{-6} dilution of the material was lethal for most of the mice inoculated intracerebrally while the 10^{-7} dilution killed most of the infected eggs.

Serial transfers were made on the chorioallantoic membrane of 10-day embryos and in the yolk sac of 5-day embryos for 6 and 7 passages, respectively (Table I). Consecutive passages were discontinued when it became apparent that the West Nile virus could be carried indefinitely in either chorioallantoic membrane or in yolk sac.

The virus became disseminated throughout the embryonated egg following inoculation by all routes investigated. This is evident from the results summarized in Table I, for the embryo contains large amounts of virus following injection of the chorioallantoic membrane, the allantoic sac, or the yolk sac. Furthermore, following introduction of virus into the yolk sac the yolk sac membrane was approximately as infectious as the embryo. Allantoic fluid contained comparatively small amounts of virus, indeed, inoculation into the allantoic sac resulted in the appearance of more virus in the embryo and allantoic membrane than in the allantoic fluid itself.

Pathological changes encountered in embryonated eggs, moribund or dead from infection with the West Nile virus, were not very conspicuous. Marked congestion of the embryo was noted following inoculation by any route. The chorioallantoic membrane was somewhat thickened and edematous following inoculation of virus on this structure, but pock-like lesions were never encountered. Histological examination of a number of the membranes and embryos failed to reveal changes other than those evident on macroscopic inspection.

Summary. The West Nile virus multiplies

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[1] Sanders, M., *Arch. Path.*, 1939, **28**, 541.

[2] Smithburn, K. C., Hughes, T. P., Burke, A. W., and Paul, J. H., *Am. J. Trop. Med.*, 1940, **20**, 471.

[3] Havens, W. P., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., *J. Exp. Med.*, 1943, **77**, 139.

TABLE I.
Comparison of Various Routes of Inoculation and the Distribution of Virus.

			Infectivity of			
Route	Passage	Age of embryo	Allantoic membrane	Allantoic fluid	Embryo	Embryo head
Allantoic membrane	1st	10 days	10^{-6}	$<10^{-2}$	10^{-5}	
	2nd	”	10^{-5}	10^{-3}		10^{-6}
	4th	”	10^{-5}			10^{-4}
	6th	”				10^{-6}
Yolk sac	1st	5 days	10^{-5}*		10^{-6}	
	2nd	”	10^{-6}*		10^{-6}	
	5th	”			10^{-5}	
	7th	”			10^{-6}	
Allantoic sac	1st	10 days	10^{-4}	10^{-3}		10^{-6}

Infectivity determined by intracerebral titration in mice.
$<$ = less than.
*Yolk sac membrane.

rapidly throughout the embryonated egg following inoculation by any of the usual routes. The embryo contains large amounts of virus while the allantoic fluid contains comparatively small amounts of the active agent. Death of the infected embryos occurs with such regularity that titration of virus can be made as well in eggs as in mice.

14090

Studies With Dogs Maintained on Diets Low in Fat.*

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The observations and data presented here constitute a part of a general study of the factors which are concerned with the nutrition of the skin. The findings that the degree of unsaturation of the serum lipids tends to be low in patients with eczema[1,2,3] and that the dietary use of fats rich in unsaturated fatty acids exerts a beneficial effect in certain patients with eczematous skin eruptions[1,3,4] together with the observations of Burr and Burr[5,6] regarding the importance of linoleic and arachidonic acids in nutrition, indicate that particular attention must be given to the unsaturated fatty acids in such a study. The dog seems to lend itself as a suitable experimental animal for observing the effects of a fat deficient diet on the general health and condition of the skin of the animal as well as for studying the distribution of the fatty acids in the various lipid fractions of the blood under these conditions.

Material and Methods. At weaning, 8 puppies born to a collie mother were given a diet low in fat composed of skim milk 73.2%, cottage cheese 7.0%, polished rice 5.7% and sucrose 14.1%. To the daily diet of each

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* Aided by grants from the National Live Stock and Meat Board through the National Research Council, and the Medical Graduate Research Fund of the University of Minnesota.

[1] Hansen, Arild E., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 160; *Am. J. Dis. Child.*, 1937, **53**, 933.

[2] Faber, H. K., and Roberts, D. B., *J. Pediat.*, 1935, **6**, 490.

[3] Finnerud, Clark W., Kesler, R. L., and Wiese, Hilda, *Arch. Dermat. and Syph.*, 1941, **44**, 849.

[4] Cornbleet, T., and Pace, E. R., *Arch. Dermat. and Syph.*, 1935, **31**, 224.

[5] Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, **82**, 345; *ibid.*, **82**, 366.

[6] Burr, G. O., *Federation Proceedings*, 1942, **1**, 224.

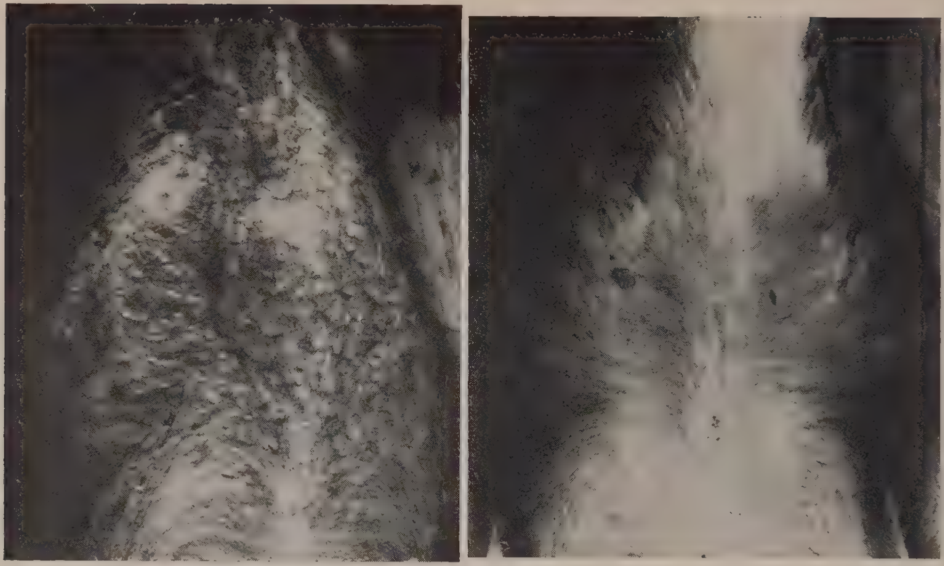

FIG. 1.

Photographs of the ventral surface of (a) puppy on low fat diet; (b) litter mate control puppy receiving 28% of the calories as lard. At this time the animals were 4½ months of age.

animal were added 0.4 g artificial bone ash mixture and 0.2 g Cowgill's salt mixture per kilogram body weight, in addition to the following: Vitamin A (concentrate in natural ester form furnished by General Mills, Inc.), 1600 units; vitamin B complex (furnished by S.M.A. Corporation) to supply individual factors in quantities recommended by Schaefer, McKibbin and Elvehjem;[7] vitamin D (Drisdol furnished by Winthrop Chemical Company), 250 units; and the weekly administration of vitamin E (Tofaxin, supplied by Winthrop Chemical Company), 25 mg, equivalent to 15 mg of alpha tocopherol. Analysis of the components of the diet showed it contained a total of 0.13% fat and furnished 1% of the total calories. The diet is essentially the same as the one used by an experimental human subject[8] and previously found to produce the typical fat deficiency symptoms in rats. Four of the litter mate puppies were used as control animals and in the diet of these, lard was substituted for 28% of the sucrose calories. Burr and his associates[5,9] showed that lard possesses high curative properties for the fat deficiency symptoms in rats.

For the serum lipid studies, blood specimens were obtained 20-24 hours following a meal. The serum was extracted with the usual 3:1 alcohol-ether mixture of Bloor and the following determinations were made in duplicate: total fatty acids by the microgravimetric technic of Wilson and Hansen;[10] the acetone soluble and the acetone insoluble fatty acids by a technic developed by Hansen;[11] the total cholesterol and the cholesterol esters by the method of Bloor[12] in which the Evelyn photo-

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[7] Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 365.

[8] Brown, Wm. R., Hansen, A. E., Burr, G. O., and McQuarrie, I., *J. Nutrition*, 1938, **16**, 511.

[9] Burr, G. O., Burr, M. M., and Miller, E. S., *J. Biol. Chem.*, 1932, **97**, 1.

[10] Wilson, W. R., and Hansen, A. E., *J. Biol. Chem.*, 1936, **112**, 457.

[11] Hansen, Arild E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 376; *ibid.*, 1939, **41**, 205.

[12] Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227; *ibid.*, 1916, **27**, 107.

TABLE I.
Comparison of Various Serum Lipid Values of Puppies Maintained on a Diet Low in Fat with Those Obtained from Litter Mate Control Animals on the Same Diet Except for the Isocaloric Substitution of Lard for Sucrose Sufficient to Supply 28% of the Total Calories.

Animal No.	Date 1942	Total fatty acids		Acetone insoluble fatty acids (phospholipid)		Acetone soluble fatty acids (cholesterol esters plus neutral fat)		Cholesterol	
		mg%	I.N.	mg%	I.N.	mg%	I.N.	Total mg%	Ester mg%
Animals on Low Fat Diet.									
19	**1-19**	311	89.8	155	93.8	142	73.9	158	100
20	1-19	235	81.5	84	84.7	111	76.9	108	62
22	2- 3*	374	84.8	148	87.3	184	79.1	160	65
22	3-31	574	92.7	303	96.0	214	90.4	273	193
20	4- 9	505	94.7	252	98.6	212	89.3	246	175
22	6- 6	480	92.3	233	96.0	—†	—	261	173
20	6-20	528	97.9	267	98.2	—†	—	382	212
19	6-22	496	95.4	239	102.4	—†	—	262	156
22	8-24	509	93.4	227	94.5	202	88.9	252	170
Avg		455	92.2	220	95.5	176	83.9	243	155
Control Dogs Receiving Lard in the Diet.									
16	1-19	409	115.5	199	113.5	184	117.0	210	147
18	1-19	461	119.6	244	115.6	221	116.5	278	188
17	2- 3*	463	98.8	213	98.3	208	97.3	246	174
15	2- 3*	416	100.8	185	104.7	204	97.4	227	173
15	3-31	658	117.1	322	115.7	275	117.6	389	308
15	4- 9	612	123.3	325	116.8	249	126.9	412	283
18	4- 9	578	115.8	309	112.0	249	116.6	354	258
18	6- 6	530	116.9	237	112.4	—†	—	346	256
15	6-20	530	117.9	207	120.0	—†	—	362	266
16	6-22	510	116.6	219	114.9	—†	—	309	180
16	8-24	501	113.5	205	110.5	219	118.0	280	166
Avg		532	117.3	252	114.6	233	118.7	327	228

*Specimens obtained at time of acute infection. (These values are not included in the averages.)

†Fractions used for further study to attempt separation into glyceride and cholesterol ester fatty acids.

electric colorimeter was used; and the iodine number of the fatty acids in each group by the use of the pyridine sulfate dibromide microreagent. The observations and results here reported were made during a 14-month interval.

Discussion of Results. With regard to the general health of the animals, the most distinct finding was that related to the skin. When the animals were 3 months of age, it was noted that the skin of the animals on the low fat diet became dry and the hair dry and coarse. Within a period of 2 weeks the character of the skin of these animals was altered even more; a definite flaky desquamation with large scales and fine scurfy specks appeared over the entire body but was most marked on the ventral surface. The skin and hair of the control animals did not show these changes. The differences in the appearance of the skin between the animals on the low fat diet and those receiving lard in the diet are shown in Fig. 1.

This abnormality in the dermal structure is similar to that observed in rats kept on a diet practically devoid of fat. The degree of skin change in the dogs however was found to vary somewhat with the season of the year and may be related to the humidity as pointed out by Burr.[6] The rate of growth of the animals in each group was essentially the same during this period. No differences were noted in activity or in the avidity with which food was consumed. A few periods of anorexia occurred but they were not confined to the dogs on the low fat diet. Repeated urinalysis failed to show either gross or microscopic hematuria. In one puppy which succumbed to an intussusception after being on the low fat diet for 4 months, no kidney lesions were found although alterations in the kidneys have been demonstrable at autopsy in 100% of the rats reared on the low fat diet according to Burr.[6] We have not as yet had the opportunity to observe the reproductive behavior of the animals on the low fat diet.

The results of the analysis of the blood lipids made at intervals on all the puppies are given in Table I.

The average value of the total fatty acids of the serum was found to be somewhat lower in the animals on the low fat diet than that of the control group. (Table I.) This difference, however, may not be significant inasmuch as in both groups of animals two-thirds of the individual values fall in the same range, namely, 400 to 600 mg per 100 cc of serum. The amount of these fatty acids in the phospholipid (acetone insoluble) fraction in both groups was greater than that present in the acetone soluble fraction. Previous studies[11] with human subjects revealed that the fatty acids in the acetone insoluble fraction of the serum tend to be in the neighborhood of 30% of the total fatty acids, whereas in these dogs, the phospholipid fatty acids comprise about one-half of the total fatty acids. It will be noted from Table I also that both the total cholesterol and cholesterol ester values are lower in the dogs reared on the low fat regimen. The values of the fatty acids in the various fractions of the serum lipids in the animals reared on a practically fat-free diet indicate that these animals are able to synthesize fatty acids. From the iodine values of the fatty acids in the various fractions as shown in Table I, it is evident that the fatty acids which are synthesized by these animals are materially different from the fatty acids present in the serum of the control dogs. The average iodine number of the total fatty acids in the low fat group was only 92.2 compared with 117.3 for that of the control dogs. There is also a difference in the degree of unsaturation of the phospholipid fatty acids in the two groups. More striking, however, is the difference in the iodine numbers of the fatty acids present in the acetone soluble fraction. The average iodine number of 83.9 for this fraction in the animals on the low fat diet stands in distinct contrast to 118.7 for the animals given lard in the diet. Since the fatty acids in the acetone soluble fraction contain both glyceride and cholesterol ester fatty acids, the resultant low iodine number of this fraction of the serum lipids of the animals on the low fat diet may be due to the synthesis of either an increased percentage of saturated fatty acids appearing as neutral fat or a decreased percentage and lower degree of unsaturation of the fatty acids combined as cholesterol ester. Preliminary experiments involving fractionation of the acetone soluble portion indicate the latter is the case. The amount of neutral fat in the serum of the two groups of animals has been found to be very nearly the same. The control animals received a plentiful supply of unsaturated fatty acids in their dietary fat and the fatty acids of the serum of these animals were found to be more unsaturated. The fractions of the serum lipids which normally contain highly unsaturated fatty acids, namely, the cholesterol esters and phospholipids, have relatively low iodine numbers in the animals on the low fat regimen which finding suggests that the dog is unable to synthesize fats of a high degree of unsaturation.

Conclusion. Distinct alterations in the appearance of the skin have been observed in puppies reared on a diet low in fat. This skin change became evident at about 3 months of age with the gradual development of a generalized flaky desquamation together with a dryness and coarseness of the hair. The skin and hair of the litter-mate puppies receiving 28% of their calories as lard in the diet remained clear and soft. Coincidental with this phenomenon marked differences in the degree of unsaturation of the fatty acids of the blood serum in the 2 groups of animals was demonstrated. The most marked difference in the iodine number of the fatty acids was found in the acetone soluble fraction, the average value being 83.9 for the animals receiving practically no fat in contrast to 118.7 for the animals receiving lard as a form of fat in the diet.

14091 P

A Biochemical Lesion of Lysine Deficiency in Man.*

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From the Harriet Lane Home of the Johns Hopkins Hospital and the Department of Pediatrics, Johns Hopkins University, Baltimore, Md.

In a previous report[1] it was shown by nitrogen balance studies that lysine is a dietary essential for man. Observations on the urine were made at that time[2] in lysine-deficient subjects, but with the exception of an increased sulfate excretion no very definite changes were encountered. The present report describes further experiments which have confirmed our previous finding of a negative nitrogen balance and have extended our studies of the urinary constituents in this deficiency. These studies have revealed a striking rise in the non-ketone organic acids of the urine in this deficiency.

The lysine-deficient diets for the 3 subjects in the present study (No. 4 ♀, No. 5 ♂ and No. 6 ♂) were similar to those described in our previous report. The protein moiety was provided by an acid hydrolysate of deaminized casein, supplemented by 1.5% *l*-tryptophane and 1% *l*-cystine; this was fed in quantities of 0.1 g N/kg/day. The total caloric intake of the subjects varied from 2400-2700 cals./day. Since these subjects had been accustomed to a somewhat larger nitrogen intake we attempted to avoid the delay in reaching an initial nitrogen equilibrium by maintaining them on a protein-free diet for two days. This was followed by a 4-day control period in which nitrogen was supplied in the quantity mentioned above in the form of a lysine free amino acid mixture which had been supplemented by 6% *l* (+) lysine. It had been planned to submit the subjects to the lysine-deficient diet for a 24-day period; however, 5 days after this regimen had been instituted, all 3 subjects complained of nausea, dizziness and hypersensitivity to metallic sounds. No objective signs were, however, discovered by clinical examinations. A study of the curves in Fig. 1 reveals that a definite negative nitrogen balance had developed in all three subjects during this brief period.

Fig. 2 illustrates the sharp rise in non-ketone organic acids[†] in the urine which developed in all 3 subjects during this period. Because of the development of these symptoms and chemical findings, it was thought desirable to return to a lysine-containing diet. They were therefore placed upon a diet in which the nitrogen was supplied by a pancreatic digest of casein (Amigen) enriched by 1% *l*-cystine. Four days of this diet sufficed to relieve the subjective symptoms; nitrogen equilibrium was restored and organic acid excretion fell to the normal level.

The lysine-deficient diet was then resumed; after 4 days 2 of the 3 subjects complained as before. The organic acid excretion rose abruptly in all 3 subjects and negative nitrogen balance recurred in the 2 subjects in which measurements were completed. At this point subject No. 4 ♀ retired from the experiment. The 2 remaining subjects were then placed for a second time on the complete casein digest (Amigen) for 3 days. Once again the nitrogen balance became more positive. The organic acid excretion dropped and the symptoms of subject No. 6 ♂ were promptly relieved. Both subjects were then returned to the lysine-deficient diet for 13 days. In this period,

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* Aided by grants from the Rockefeller Foundation, Merck and Company, E. R. Squibb and Sons and the Nutrition Foundation.

[1] Albanese, A. A., Holt, L. E., Jr., Brumback, J. E., Jr., Hayes, M., Kajdi, C., and Wangerin, D. M., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 728.

[2] Albanese, A. A., Kajdi, C., and Wangerin, D. M., *Fed. Proc.*, 1942, **1**, 97.

[†] This was determined by the method of Van Slyke and Palmer.[3] The absence of ketone bodies, which were tested for independently, permits the designation of "non-ketone" organic acids.

[3] VanSlyke, D. D., and Palmer, W. W., *J. Biol. Chem.*, 1920, **41**, 567.

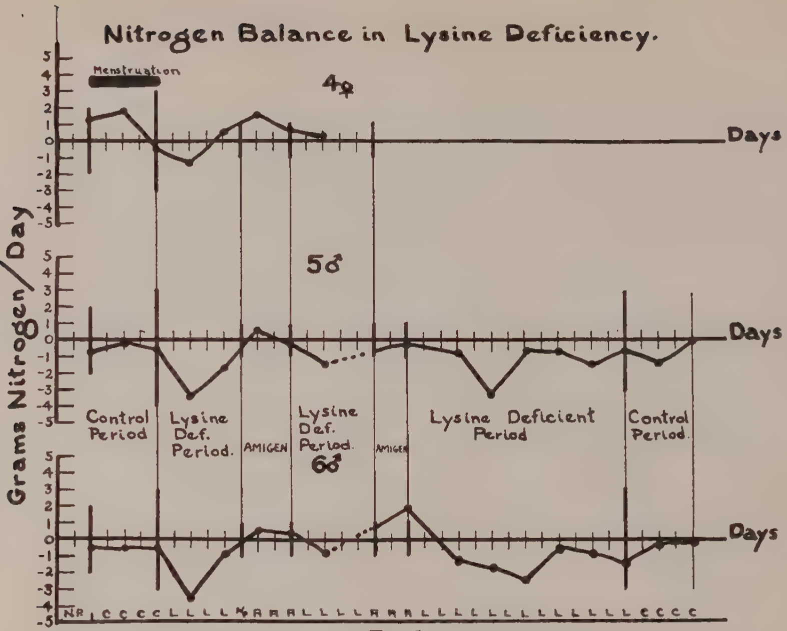
Nitrogen Balance in Lysine Deficiency.
Menstruation
4♀
5♂
6♂
Grams Nitrogen/Day
5
4
3
2
1
0
-1
-2
-3
-4
-5
Days
Control Period
Lysine Def. Period.
AMIGEN
Lysine Def. Period.
AMIGEN
Lysine Deficient Period
Control Period.
NR C C C C L L L L ½A A A L L L L A A A L L L L L L L L L L L L L C C C C

FIG. 1.

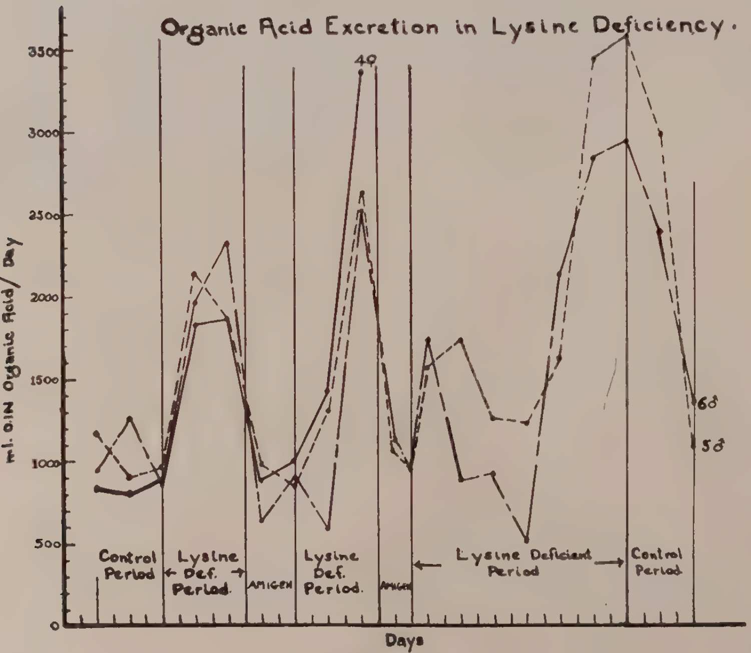
Organic Acid Excretion in Lysine Deficiency.
ml. 0.1N Organic Acid/Day
3500
3000
2500
2000
1500
1000
500
0
4♀
6♂
5♂
Control Period
Lysine ← Def. → Period.
AMIGEN
Lysine Def. Period.
AMIGEN
← Lysine Deficient → Period
Control Period
Days

FIG. 2.

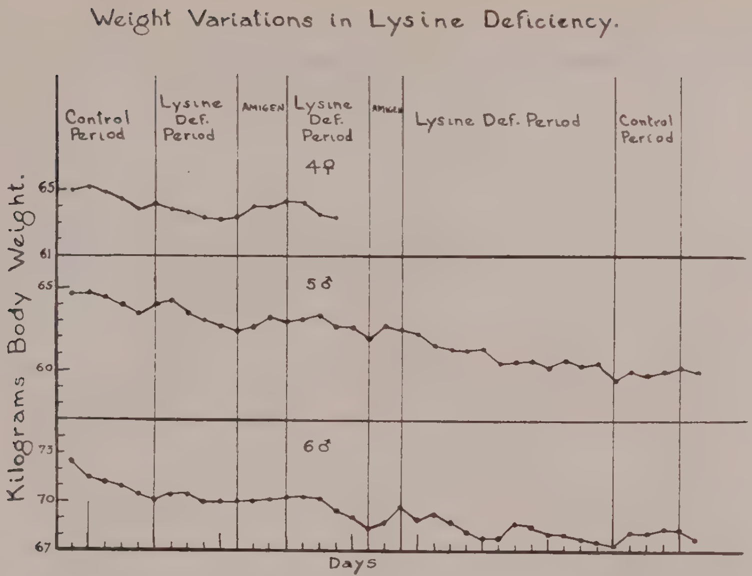

FIG. 3.

although a negative balance was immediately established and sustained, the organic acid excretion rise was delayed and none of the previous symptoms were reported. During the 4-day post-control period in which the lysine-deficient diet was supplemented with *l* (+) lysine, the negative nitrogen balance was restored and the organic acid output dropped to the normal level. The variations in weight of the subjects during the course of the experiment are shown in Fig. 3.

It would appear from these experiments that a high urinary excretion of organic acid is regularly induced by the lysine-deficient state in humans. The symptoms of nausea, dizziness and auditory hypersensitivity are apparently associated with this phenomenon. They are, however, much more conspicuous in some individuals than in others, for they were not observed in our previous studies of this deficiency.[1] It is regrettable that the organic acid output was not followed at that time.

The increased output of organic acids described above appears to be a biochemical lesion characteristic of lysine deficiency. We have not encountered it in parallel studies of deficiencies of other single amino acids—tryptophane, methionine and cystine, and it is clear from observations in the literature that a negative nitrogen balance does not *per se* give rise to this finding. It is, however, of interest to note that increases of non-ketone organic acids in the urine have been found in pneumonia,[4,5,6,7] nephrosis,[8] malnutrition,[9] postoperative acidosis,[10] premature infants[11] and in rickets of the Fanconi type.[12,13] A possible abnormality of lysine catabolism in these states is a matter upon which one can only speculate at the present time. The nature of the organic acid excreted by lysine-deficient subjects is now under investigation.

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[4] Palmer, W. W., *J. Exp. Med.*, 1917, **26**, 495.

[5] Clausen, S. W., *Arch. Int. Med.*, 1925, **35**, 57.

[6] Holten, C., *Ibid.*, 1926, **38**, 489.

[7] Greenwald, I., *J. Biol. Chem.*, 1930, **85**, 447.

[8] Perlzweig, W. A., personal communication.

[9] Utheim, K., *Am. J. Dis. Child.*, 1921, **22**, 329.

[10] Jeans, P. C., and Tallerman, K. H., *Am. J. Dis. Child.*, 1924, **28**, 253.

[11] Branning, *J. Clin. Invest.*, 1942, **21**, 105.

[12] Guild, H. G., Pierce, J. A., and Lilienthal, J. L., *Am. J. Dis. Child.*, 1937, **54**, 1186.

[13] McCune, D. J., Mason, H. H., and Clarke, H. T., *Am. J. Dis. Child.*, 1943, **65**, 81.

14092

Composition of the Cervical Sympathetic Trunk.*

James O. Foley.

From the Department of Anatomy, University of Alabama.

Introduction. The cervical sympathetic trunk has generally been regarded as being composed solely of myelinated preganglionic axons.[1] It has been shown recently by Foley and DuBois[2] that this view is no longer tenable; for, not only are many of the nerve fibers of the cervical sympathetic trunk of the cat, dog, and rat unmyelinated, but nerve fibers persist in the trunk of the cat after the preganglionic axons have been eliminated by sectioning the roots or removing the ganglia of the first nine thoracic spinal nerves. In this report, however, the authors recognized that the number of persistent axons which was reported should be accepted with reservations since regeneration might have contributed to some of them, a possibility favored by the rather long post-operative survival times employed in some cases. Accordingly, further experiments, in which the postoperative survival times were reduced, have been devised with the intention of confirming or revising the previous results.

Materials and Methods. The preganglionic axons were eliminated from the cervical sympathetic trunks of 13 cats by three methods: (a) section of the dorsal and ventral roots of the first 9 thoracic spinal nerves, (b) removal of the dorsal root ganglia of the first 9 thoracic spinal nerves, or (c) severance of the rami which pass to the stellate ganglion from the upper thoracic nerves and simultaneous removal of a segment of thoracic sympathetic trunk below the stellate ganglion.† In 5 animals the surgery was performed on the right side while in 8 others it was done on the left.

The time allotted for degeneration of the preganglionic nerve fibers in the cervical sympathetic trunk after both rhizotomy and ganglionectomy of the spinal nerves was 21 days or less. A period of 21 days is an adequate maximum interval for degeneration, there being no histological evidence of regenerating axons in the degenerated nerves of the animals which have been terminated at the end of this postoperative survival period. On the other hand, histological examination of the degenerate cervical sympathetic trunks of cats, in which the preganglionic axons have been eliminated by combined sympathectomy and section of the rami of the stellate ganglion, has shown that regeneration often occurs if the animals are allowed to live as long as 21 days. Here the favorable postoperative survival time appears to be from 10 to 14 days. After appropriate periods of time the animals were sacrificed and the degenerated and normal nerves were secured and prepared for histological study and quantitative analysis by methods described previously by the writer.[2]

Results. The total number of axons in the 18 cervical sympathetic trunks of the unoperated sides (Table I) ranges from 5404 to 10127 and averages 7422. The differential count shows that the myelinated fibers outnumber (average 67% myelinated) the unmyelinated fibers in 15 of the 18 normal trunks (Table I‡). In 4 of the 15 (cats 111, 6B, 10B, and 120) the margin of excess is not great (average 55% myelinated). In 3 of the 18 (cats 10, 3 and 117), moreover the myelinated fibers comprise, respectively 43, 48 and 37% of the total number of axons enumerated in each normal trunk. In only one instance (cat 3B, 96%) is the normal trunk almost completely made up of myelinated fibers.

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* Aided by a grant from the Research Fund of the University of Alabama.

[1] Billingsley, P. R., and Ranson, S. W., *J. Comp. Neur.*, 1918, **29**, 359.

[2] Foley, James O., and DuBois, F. S., *J. Comp. Neur.*, 1940, **72**, 587.

† The author wishes to thank Doctor Allen D. Keller of the Department of Physiology for performing most of the combined sympathectomy and ramisectomy operations.

TABLE I.

			Nerve fibers in cervical sympathetic trunk after ablation of its thoracic preganglionic axons					
			Operated side			Unoperated side		
Cat No.	Surgery	Survival time in days	Total axons	Myelinated axons	Unmyelinated axons	Total axons	Myelinated axons	Unmyelinated axons
			%				%	%
2B	RS	10	311 (5)+	13	298	6854	4954 (72)‡	1900 (24)*
1B¶	RS	10	111 (2)+	5	106	7261	5031 (70)‡	2230 (30)*
5B¶	RS	12	126 (2)+	24	102	8405	6039 (72)‡	2366 (27)*
9B¶	RS	12	554 (7)+	5	549	7549	5283 (70)‡	2266 (25)*
111 ¶	RS	13	1473 (24)+	2	1471	6124	3508 (57)‡	2616 (25)*
6B¶	RS	14	285 (3)+	3	282	8799	5087 (58)‡	3712 (40)*
3B	RS	14	221 (3)+	7	214	7312	7036 (96)‡	276 (1)*
4B	RS	14	386 (5)+	67	319	8127	5592 (69)‡	2535 (29)*
10B	RS	14	283 (3)+	2	281	8608	4483 (52)‡	4125 (46)*
120	G	14	490 (6)+	38	452	8083	4207 (52)‡	3876 (44)*
10	G	20	2619 (33)+	8	2611	7983	3400 (43)‡	4583 (37)*
3	R	21	410 (4)+	4	406	10127	4841 (48)‡	5286 (50)*
117	G	21	420 (6)+	7	413	7394	2702 (37)‡	4692 (61)*
148†	R	37	2535 (38)+	164	2371	6681	3705 (55)‡	2976 (15)*
149†	R	49	1149 (17)+	49	1100	6927	5555 (80)‡	1372 (5)*
147†	R	94	1535 (28)+	262	1273	5404	4299 (79)‡	1105 (0)*
144†	G	104	1918 (34)+	204	1714	5622	3414 (61)‡	2208 (13)*
142†	G	107	2117 (33)+	101	2016	6342	3801 (60)‡	2541 (12)*

† = Foley, James O., and DuBois, Franklin S., *J. Comp. Neur.*, 1940, **72**, 587.

+ = % of total axons of the nerve of the unoperated side. Subtract from 100% for determination of total preganglionic axons.

‡ = % of total axons of unoperated side which is myelinated.

* = % of preganglionic axons which is unmyelinated. Method of determination described in previous publication.[2]

¶ = Surgery done on right side, all others on left.

RS = Combined sympathectomy and section of rami of stellate ganglion.

R = Rhizotomy of the first 9 thoracic spinal nerves.

G = Ganglionectomy of the first thoracic nerves.

The degenerated sympathetic trunks[‡] of the cats which lived for 10 to 21 days after surgery contain from 111 to 2619 axons and these represent 2 to 33% (Table 1+) of the total number of nerve fibers in the intact trunk of the unoperated side. The average number of axons in these 13 degenerated nerves is 592, and this constitutes 8% of the total average number of nerve fibers of the unoperated trunks. On the average, more axons were found in the degenerated left (623 fibers) than in the degenerated right (510 fibers) (Table I¶) cervical sympathetic trunk. The differential count of the residual fibers (Table I) in the degenerated trunks of the animals with short survival times (10 to 21 days) shows that relatively few (average 2%) of the total number of persistent axons are myelinated, most are unmyelinated.

A comparison of the number of myelinated and unmyelinated fibers in the operated trunks (Table I) shows a significant and constant relationship to the postoperative survival time. On the average, there are approximately 10 times more myelinated fibers (156 as contrasted with 15) and almost 4 times more unmyelinated fibers (1695 as contrasted with 460) in the degenerated sympathetic trunks of the cats with long postoperative survival times (37 to 107 days) than in those of the animals with shorter postoperative survival times (10 to 21 days). The greater number of nerve fibers in the degenerated nerves of the animals which lived longer than 21 days after surgery indicates that significant numbers of regenerated axons are present in the degenerated sympathetic trunks of the cats with long postoperative survival periods.

[‡] This statement applies at all times to the main body of the trunk and does not include those fascicles which can be followed into it from the vagus and superior cervical ganglion.[2]

In 11 of the 13 sympathetic trunks, described here for the first time, the surgical procedures removed an average of approximately 95% (Table I+) of the total number of axons while in the remaining two 67 and 76% were removed. The present experiments indicate that the majority of nerve fibers in the cervical sympathetic trunk are preganglionic axons from thoracic levels of spinal cord. The deleted preganglionic axons are predominantly myelinated since the myelinated axons represent, in most cases, the most numerous type of nerve fiber in the cervical sympathetic trunk and almost all of them are removed by the surgery employed (Table I). Significant numbers of preganglionic axons, however, are unmyelinated and these range from 1 to 61% and average 34% (Table I*).

Discussion. The results obtained in the present research support the general thesis of the previous report;[2] namely, that large numbers of the preganglionic fibers of the cervical sympathetic trunk are unmyelinated and that variable numbers of axons remain in the cervical sympathetic trunk after the preganglionic fibers have been eliminated. The present findings, however, indicate that the earlier results[2] were complicated by regeneration. The number of residual axons reported previously were too high since their normal number was augmented by regeneration. Furthermore, the percentages of unmyelinated preganglionic axons which were recorded were too low since the majority of the regenerated axons were unmyelinated and their presence adversely affected the calculation[2] of the number of unmyelinated preganglionic axons.

Caution should be exercised in drawing conclusions on the ratio which exists between preganglionic and postganglionic neurons when such conclusions are based only on an enumeration of the myelinated axons since the present observations indicate that significant numbers of the axons in the cervical sympathetic trunk are unmyelinated. Confirmation of this contention, previously indicated[2] and now reemphasized, is furnished by the recent findings of Wolf.[3] This investigator has demonstrated that the ratio between preganglionic axons in the cervical sympathetic trunk and cells in the superior cervical ganglion is materially reduced, from the ratio reported previously,[1] when the unmyelinated fibers of the cervical sympathetic trunk are taken into consideration. Furthermore, the observation by the writer that not all of the nerve fibers in the cervical sympathetic trunk are preganglionic axons indicates the desirability of a further revision of the ratios between preganglionic and postganglionic neurons in the cervical sympathetic trunk.

Summary. The total number of nerve fibers enumerated in 18 normal sympathetic trunks, varied from 5404 to 10127. In 15 of the 18 nerves the myelinated exceeded the unmyelinated axons but in the majority of the 15 nerves the excess of myelinated over unmyelinated axons is not great. In only one instance was the trunk almost completely composed of myelinated fibers (96%).

A variable number of nerve fibers (111 to 2619) remains in the cervical sympathetic trunk after its preganglionic axons have been eliminated. These residual fibers comprise 2 to 33% of the total number of nerve fibers in the cervical sympathetic trunk and the majority of them are unmyelinated.

The majority (67 to 98%) of the nerve fibers in the cervical sympathetic trunk are preganglionic axons from thoracic levels of spinal cord. Considerable variation exists in the ratio between myelinated and unmyelinated preganglionic axons in different cats, the unmyelinated ranging from 1 to 61% and averaging 34%.

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[3] Wolf, George A., *J. Comp. Neur.*, 1941, **75**, 235.

14093

Hepatic Vascular Occlusion Following Lethal Doses of Salmine Sulfate.

W. B. Shelley and R. Tarail. (Introduced by M. B. Visscher.)
From the Department of Physiology, University of Minnesota, Minneapolis.

Previous studies have shown that the intravenous injection of salmine sulfate (a protamine) causes anaphylactoid shock.[1] The production of emboli may play a role in this phenomenon.[2] This hypothesis was based on 1. The demonstration *in vitro* of salmine-induced protein precipitation and in some cases hemagglutination in the blood of various species. 2. The addition of salmine sulfate to blood perfusing the normal liver or lung resulted in obstruction to flow. 3. Similar addition of salmine sulfate to saline solutions perfusing these organs failed to diminish flow.

In no experiments previously reported has the blood flow through any vascular bed been studied in animals killed with lethal intravenous doses of protamine. The present experiments were undertaken with a view to testing *in vivo* the assumed vascular occlusive effects of salmine sulfate. Since the nature of the postulated emboli appeared to preclude their direct microscopic demonstration, liver perfusion studies were made after administering protamine solutions intravenously. The rat and guinea pig were chosen because of the difference in the effect of salmine on their bloods.[2] Although salmine sulfate causes a precipitate of plasma protein in the blood of both, hemagglutination is produced only in the rat.

Method. Rats (220-260 g) and guinea pigs (225-300 g) were anesthetized by the intraperitoneal injection of 5 mg of 5% pentobarbital sodium/100 g of body weight. With the onset of respiratory failure produced by the injection of 10-50 mg of 2% salmine sulfate* per 100 g into the portal vein of the anesthetized animal, cannulation of the portal vein and the thoracic inferior vena cava was performed. Physiological saline at 37°C ± 1°C was delivered from a Mariotte flask to the portal cannula at pressures varied progressively from 2-42 cm of saline. The rate of flow of the perfusion fluid at various pressures has been employed as the criterion of the occlusive effect. Respiratory failure was induced in the control animals by ether, pentobarbital, or pneumothorax.

Results and Discussion. The results of experiments on 22 animals are given in Table I. Perfusion rates in salmine-treated rats and guinea pigs are significantly and consistently lower than in the controls. The observations with portal pressures of 4 to 8 cm of saline should be especially emphasized, for these constitute the range of normal pressures.[3] At portal pressures of 4 and 8 cm, average control rates of 5 and 11 cc of saline per minute were observed in the rat. In the treated animals no appreciable flow occurred at these pressures. In the rats given 50 mg per 100 g no flow could be induced using pressures as high as 42 cm. In the guinea pig comparable but less pronounced reduction in flow occurred. It can be concluded that lethal intraportal doses of salmine sulfate produce a vascular occlusive effect on the hepatic circulation of the animals studied.

The difference between the degree of vascular occlusion in the rat as compared to the guinea pig parallels the difference noted in *in vitro* studies of the effect of salmine sulfate on the blood of these animals. Both hemagglutination and protein precipitation occur in the rat, whereas only protein precipitation was noted in the guinea pig.

Among the factors which may be causally related to this impedance to flow are the lodgment of multiple small emboli, local cellular edema, and/or local vasospasm. The present studies do not directly differentiate the relative importance of these factors. But when

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[1] Thompson, W. H., *Trans. Roy. Acad. Med. in Ireland*, 1900, **18**, 426.

[2] Shelley, W. B., Hodgkins, M. P., Visscher, M. B., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 300.

* Kindly placed at our disposal by Eli Lilly and Company of Indianapolis, Indiana.

[3] Trowell, O. A., *J. Physiol.*, 1942, **100**, 432.

TABLE I.
Perfusion Rates Through Portal Circulation of Isolated Livers of Rats and Guinea Pigs after Intraportal Administration of Salmine Sulfate at Various Dose Levels.

Animal	No. of animals	Dose of salmine sulfate mg/100 g	Perfusion flow rate—cc/min Perfusion pressure:					
			2 cm	4 cm	8 cm	16 cm	32 cm	42 cm
Rat	4	Control	1	5	11	25	50	50
	3	10	0	0	1	4	26	39
	2	20	0	0	0	1	15	27
	2	50	0	0	0	0	0	0
Guinea Pig	4	Control	4	8	20	34	51	62
	3	10	0	1	9	17	45	70
	2	20	0	1	5	17	33	60
	2	50	0	0	4	17	39	51

considered in conjunction with our previous observations, they are consistent with the hypothesis that embolus formation plays a role in the toxicity of intravenously administered salmine sulfate. No studies have been made to evaluate the importance of direct histotoxic effects.

Conclusion. 1. Vascular occlusion, as shown by perfusion studies, occurred in the livers of rats and guinea pigs receiving lethal doses of salmine sulfate. 2. This effect was of greater magnitude in the rat than in the guinea pig.

14094

Metabolism of Glycolic and Glyoxylic Acids.*

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In the metabolism of both glycine and fatty acids it has been proposed that 2 carbon acids are formed which in turn take part in the formation of glucose or the acetone bodies. The purpose of this communication is to present further information on the metabolism of 2 compounds, glycolic acid ($HOCH_2$—COOH) and glyoxylic acid (O:HC—COOH) either of which might be involved in the above transformations.

Methods. Three types of experiments were performed. (1) Liver glycogen formation studies were done by administering the test substances by stomach tube to adult male rats which had been previously fasted for 48 hours. Glycogen was determined by the method of Good, Kramer and Somogyi.[1] (2) Urine acetone body excretion studies were made by administering the test substance by stomach tube over a 4-day period to fasting adult male rats which previous to fasting had been maintained on a high fat-low protein diet. Daily urine collections were made from individual rats and acetone bodies were determined by the method of Van Slyke.[2] (3) Blood acetone bodies were studied in rats which were taken from the stock diet and immediately fed the test substances by stomach tube. The rats were anesthetized with amytal and bled from the large abdominal vessels and blood acetone bodies were determined by the method of Barnes and Wick.[3]

Results. Liver glycogen after administering either glycolic acid or sodium glycolate, the

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* Aided by a grant from the Rockefeller Foundation.

[1] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1917, **32**, 455.

[3] Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, 1939, **131**, 413.

TABLE I.
Liver Glycogen at Various Time Intervals after Feeding Glycolic Acid to Fasting Male Rats.

Substance fed*	Time, hr	No. of rats	Body wt, g	Liver wt, g	Liver glycogen, %
		Experiment I.			
	0	2	97	3.61	0.23
1M glycolic acid	4	2	105	3.68	0.29
” ” ”	8	1	100	3.36	0.97
” ” ”	12	1	93	3.00	0.02
” ” ”	16	1	95	3.38	0.57
” ” ”	22	1	103	3.60	0.94
		Experiment II.			
	0	2	114	4.19	0.18
1M sodium glycolate	6	2	113	3.84	0.15
” ” bicarbonate	6	2	113	3.95	0.09
” ” glycolate	12	2	114	3.95	0.18
” ” bicarbonate	12	2	114	4.23	0.51
” ” glycolate	20	1	115	4.45	0.82
” ” bicarbonate	20	1	112	4.13	0.39

*In Experiment I: Male rats were fasted for 48 hours and then fed by stomach tube 1 cc of 1M glycolic acid per square decimeter body surface. The rats were then killed at the stated intervals.

In Experiment II: Male rats were fasted for 48 hours and then fed by stomach tube 1 cc of 1M sodium salts of either carbonic acid or glycolic acid per square decimeter body surface. Four hours later they received by stomach tube 1 cc of 0.5 M sodium salts of the 2 acids respectively per square decimeter body surface.

Body surface was calculated by the formula of Carman and Mitchell.[12]

TABLE II.
Urine Acetone Bodies After Administering Glycolic Acid to Fasting Adult Male Rats.

				Urine acetone bodies per sq.dm. body surface per day (calculated as acetone)			
Substance fed	No. of rats	Body wt g	Body surface sq cm	1st mg	2nd mg	3rd mg	4th day mg
		Experiment III.*					
0.5M sodium bicarbonate	4	205	395	1.3	6.3	10.5	5.6
0.5M sodium glycolate	4	203	391	1.0	6.2	14.0	8.7
		Experiment IV.†					
0.75M sodium bicarbonate	5	242	441	2.5	8.2	15.3	12.3
0.75M sodium glycolate	5	241	438	4.8	4.9	19.7	10.2

*High fat-low protein diet containing 4% yeast was fed for 14 days before experimental fasting period was begun. 1.0 cc of either 0.5M sodium bicarbonate or sodium glycolate per sq.dm. body surface was fed by stomach tube twice daily.

†High fat-low protein diet containing the known required synthetic B vitamins, but without choline was fed for 10 days prior to the experimental fasting period. 1.0 cc of either 0.75M sodium bicarbonate or sodium glycolate per sq.dm. body surface was fed by stomach tube twice daily.

latter controlled with sodium bicarbonate, was followed for 20 hours. The results which are presented in Table I show that under the conditions of the experiment, glycolic acid is not converted into glycogen. Fed under similar conditions the same dose of glycine results in a marked liver glycogen increase. Therefore, the dose of glycolic acid employed here was adequate. A similar experiment was attempted with glyoxylic acid, but this compound was found to be too toxic in the amounts necessary for this study.

Upon administering glycolic acid urine acetone body excretion shows a negative response (Table II). If the glycolic acid had been ketogenic it would have been observed in these experiments for in the same concentration acetic acid is ketogenic.[4]

In Table III are presented data on the ketogenic activity of both glycolic and gly-

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[12] Carman, G. G., and Mitchell, H. H., *Am. J. Physiol.*, 1926, **76**, 380.

[4] MacKay, E. M., Barnes, R. H., Carne, H. O., and Wick, A. N., *J. Biol. Chem.*, 1940, **135**, 157.

TABLE III.
Blood Acetone Bodies After Administering Either Glycolic Acid or Glyoxylic Acid to Fed Adult Male Rats.[*]

Exp. No.	Substance fed	No. of rats	Body wt, g	Time, hr	Blood acetone bodies calculated as acetone mg%
	Glycolic Acid Experiments.				
5		3	211	0	0.11
5	1M sodium bicarbonate	3	216	6	0.00
5	1M sodium glycolate	3	208	6	0.00
5	1M sodium bicarbonate	3	208	12	1.00
5	1M sodium glycolate	3	208	12	0.35
6	Controls	4	233	[†]	0.71
6	0.5M glycolic acid	4	227	[†]	0.42
	Glyoxylic Acid Experiments.				
7		2	228	0	0.48[‡]
7	0.5M sodium bicarbonate	3	233	6	0.43
7	0.5M sodium glyoxylate	3	232	6	1.25
7	0.5M sodium bicarbonate	3	243	12	0.80
7	0.5M sodium glyoxylate	3	237	12	1.50
8	Controls	3	275	[†]	1.26
8	0.5M sodium glyoxylate	3	300	[†]	3.43

[*] In experiments 5 and 7, 1 cc of the test solution per square decimeter body surface was administered by stomach tube. After the stated time intervals the rats were killed and blood acetone body determinations were made. All experiments included in this table were performed on rats taken directly from a stock diet.

[†] In experiments 6 and 8, 5.0 cc of the test solutions were administered by stomach tube; 4 hours later a second dose of 3.0 cc was given. Three hours after the second dose the animals were killed and blood acetone body determinations were made.

[‡] Not included in this average was one rat having a fasting blood acetone body concentration of 2.30 mg per 100 cc. This single value was higher than that of any other animal in experiment 7.

oxylic acids in fed animals. Glycolic acid gave no evidence of acetone body formation while glyoxylic acid resulted in a rise in blood acetone bodies in every instance. Control determinations showed that glyoxylic acid *per se* did not react with the Deniges reagent.

Discussion. Both of these substances have been considered as intermediates in the metabolism of the amino acid glycine. For instance Griffith[5] has found that the retardation of growth that is observed when large quantities of benzoic acid are fed is overcome by feeding glycolic acid. Although it is now established that the administration of glycine causes the deposition of glycogen in the fasted animal,[6] Greenwald[7] was unable to increase the urinary glucose of a phlorhizinized dog with glycolic acid, and the present investigation shows a lack of glycogenic effect of the acid in the fasted rat.

These observations did not include a study of glycogen formation from glyoxylic acid, but this transformation would appear most unlikely as this substance was shown to be ketogenic. Furthermore MacKay (personal communication) has found that glycine administration under the same conditions as were employed in our experiments does not result in any rise in blood acetone bodies. It would appear that neither glycolic nor glyoxylic acids are glycogen formers and yet these two compounds are the usual "text-book" representations of the intermediates formed in the conversion of glycine to glucose.

The observation that acetic acid causes an increased production of acetoacetic acid in the perfused liver led Toenniessen and Brinkmann[8] to propose the hypothesis that acetic acid is split from fatty acids by β-oxidation and that this acetic acid then condenses with a similar molecule to form acetoacetic acid. MacKay and his coworkers have given additional evidence for an increased production of acetone bodies in fasted rats upon feeding acetic acid.[4] By the use of heavy carbon labeling of acetic acid final proof of its conversion into the acetone bodies was established by Swendseid *et al.*[9] Although acetic acid is ketogenic in the fasting animal it has been found by MacKay (personal communication) and also in this laboratory that it will not form acetone bodies in the fed animal. MacKay, Wick and Barnum[10,11] in studying acetone body formation from the odd carbon-fatty acids, and short chain even numbered carbon fatty acids have found that if the chain contains 4 to 10 carbons, acetone bodies are formed even in the presence of normal liver glycogen. This gives ample proof that ketogenesis can, under certain conditions, go on in the fed animal. Furthermore, the fact that valeric acid forms acetone bodies under such conditions gives additional evidence that the acetone body formation takes place through a 2-carbon compound condensation.

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[5] Griffith, W. D., Proc. Soc. Exp. Biol. and Med., 1930, **28**, 216.

[6] MacKay, E. M., Wick, A. N., and Carne, H. O., *J. Biol. Chem.*, 1940, **132**, 613.

[7] Greenwald, I., *J. Biol. Chem.*, 1918, **35**, 461.

[8] Toenniessen, E., and Brinkman, E., *J. Physiol. Chem.*, 1938, **252**, 169.

There are 4 possible 2-carbon acids that may be formed by β-oxidation of a fatty acid. These are acetic, glycolic, glyoxylic, and oxalic acids. In order to fulfil the qualifications of the modified β-oxidation hypothesis, the 2-carbon acid must be capable of acetone body formation in the fed animal. Experiments which have been performed in this laboratory have shown that oxalic acid is too toxic for a study of acetone body formation. Neither acetic nor glycolic acids will form acetone bodies in the fed animal which leaves glyoxylic as the only possible intermediate of this type. It is possible that a preliminary reduction or some other reaction of the fatty acid prior to its cleavage might take place, and in such an event other intermediates might exist. However, as a working hypothesis it is proposed that in the catabolism of fatty acids glyoxylic is formed and under the proper conditions this compound is converted into the acetone bodies by the liver.

Summary. It has been shown that in the fasted rat glycolic acid does not form glycogen nor does it form acetone bodies in the fed or fasted rat. Glyoxylic acid causes the formation of acetone bodies in the fed rat. From this evidence it has been concluded that neither glycolic nor glyoxylic acids are intermediates in the transformation of glycine to glucose in the animal body.

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[9] Swendseid, M. E., Barnes, R. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, 1942, **142**, 47.

[10] MacKay, E. M., Wick, A. N., and Barnum, C. P., *J. Biol. Cehm.*, 1940, **135**, 183.

[11] MacKay, E. M., Wick, A. N., and Barnum, C. P., *J. Biol. Chem.*, 1940, **136**, 503.

14095

Correlation of Extent of Tuberculosis with Amount of Polysaccharide in the Serum.*

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An intensive study has been made in recent years of tuberculous sera, with the objective of detecting the presence of antigen, antibody or any substance which may be characteristic of the disease. The primary approach has been based upon observation of the electrophoretic diagrams of sera by means of the Tiselius technic.

It was shown[1] that during the development of tuberculosis in the rabbit the beta globulin fraction increased in proportion to the extent of the disease. In the human being, on the other hand, the consistent change[1] was a progressive increase in the gamma globulin. Increases in alpha and beta globulin fractions

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* Aided by a grant from the Committee on Medical Research of the National Tuberculosis Association.

[1] Seibert, F. B., and Nelson, J. W., *Am. Rev. Tuberc.*, 1942, **47**, 66.

TABLE I.
Amount of Polysaccharide in Fractions of Serum.

Sera fractionated	% of polysaccharide in the Albumin	Gamma globulin	Whole serum (dialyzed)
A.H.—Far advanced tuberculosis	3.3	2.4	2.4
P.R.—Pleural fluid, far advanced tuberculosis	3.1	2.4	
M.M.—Extensive sarcoidosis	2.2	1.2	
No. 78—Normal rabbit serum	1.0	1.0	

also frequently occurred but not with the same degree of regularity as was the case with the gamma globulin.

An explanation of the cause of the rise in any of these components would be of value in the understanding of the disease. The possibility of the presence of antibodies immediately presents itself in connection with the rise in the gamma globulin, especially in view of the fact that antibodies to the tuberculin protein have been definitely demonstrated[2] to be present in this fraction in sensitized rabbits. However, there could not be shown to be sufficient antibody to account for the total rise in the gamma globulin.

In seeking for other explanations of the rise, one is impressed with the idea that if tuberculin polysaccharide were found free in the serum, it would migrate in electrophoresis with approximately the same mobility as the gamma globulin.[3] Analyses were, therefore, made of the polyssacharide content of the albumin and the gamma globulin fractions isolated from several sera by means of electrophoresis.

The polysaccharide was determined directly on the protein solutions, without preliminary hydrolysis, by means of the carbazole reaction,[4] as outlined previously,[5] using the Evelyn colorimeter. Table I shows the percentage of polysaccharide in the isolated albumin and gamma globulin fractions. These results are in the same range of values reported by other investigators. For example, Kekwick[6] found that the A fraction of human serum albumin contained 1.95% polysaccharide, and Hewitt[7] stated that the carbohydrate content of the total albumin fraction of serum was usually about 2.8%. Blix, Tiselius and Svensson[8] found 1.1 to 1.2% mannose in the albumin and 3.6 to 3.0% in the gamma globulin fraction of normal sera. In a pneumonia serum there was considerably more carbohydrate in the albumin (5.8%). Nilsson[9] reported a rise in the amount of carbohydrate bound to protein in the albumin as well as globulins of sera in pneumonia.

From the results in Table I it is clear that the amount of polysaccharide in both albumin and gamma globulin was higher in the serum (A.H.) and the pleural fluid (P.R.) both from cases of far advanced tuberculosis, than in the serum (M.M.) which was from a severe case of sarcoidosis, or than in the serum (No. 78) of a normal rabbit. It also becomes clear from these results that the obvious rise usually found in the gamma globulin fraction of sera of tuberculous patients is not due primarily to the presence of tubercle bacillus polysaccharide. Instead the polysaccharide which is present appears to be able to attach itself to all the serum protein fractions and possibly to the largest extent to the albumin. It does not easily separate from the proteins since these fractions have all been dialyzed before analysis. The serum (A.H.), when subjected undiluted to electrophoresis at pH 7.6 $\mu = 0.1$, showed almost immediately a definite separation of

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[2] Seibert, F. B., and Nelson, J. W., *J. Am. Chem. Soc.*, 1943, **65**, 272.

[3] Seibert, F. B., and Watson, D. W., *J. Biol. Chem.*, 1941, **140**, 55.

[4] Dische, Z., *Mikrochemie*, 1930, **8**, 4.

[5] Seibert, F. B., *J. Biol. Chem.*, 1940, **133**, 593.

[6] Kekwick, R. A., *Biochem. J.*, 1938, **32**, 552.

[7] Hewitt, L. F., *Biochem. J.*, 1936, **30**, 2229.

[8] Blix, G., Tiselius, A., and Svensson, H., *J. Biol. Chem.*, 1941, **137**, 485.

[9] Nilsson, I., *Bioch. Zeits.*, 1937, **291**, 254.

TABLE II.
Correlation of Carbohydrate Content of Sera with Diagnoses.

Diagnoses	Symptoms	Total Carbohydrate, mg%	Glucose, mg%	Polysaccharide, mg%
F.S. Normal		194	97	97
M.S. ”		194	82	112
J.N. ”		181	51	130
H.T. ”		158	70	88
I.W. ”		157	57	101
C.C. ”		185	84	101
J.R. ”		195	75	120
W.L. ”		200	80	120
F.V. ”		209	82	127
W.S. ”		187	89	98
R.M. ”		216	92	124
T.W. ”		204	78	126
E.R. ”		211	78	133
C.E.B. Reinfection tuberculosis—Minimal	None	226	94	132
T.G. ” ” ”	Moderate	205	76	129
C.T. ” ” ” ; stationary	Slight	213	81	132
V.O. ” ” ” ; bilateral	None	208	78	130
L.J. ” ” ”	”	226	82	144
W.A. ” ” ” ”	”	227	71	156
P.D. ” ” ” ”	”	275	114	161
J.U. ” ” ”	”	238	76	162
H.W. ” ” ” ”	”	230	58	172
J.H.Jr. ” ” ” ”	”	263	79	184
J.H. Reinfection tuberculosis—moderately advanced (rarefaction cleared)	”	231	115	116
E.W. Reinfection tuberculosis—moderately advanced, cavity closing (?), bilateral	Slight	229	85	144
A.N. Reinfection tuberculosis—moderately advanced	None	225	78	147
P.B. Reinfection tuberculosis—moderately advanced; progressive; bilateral	Hemoptysis	227	78	149
J.B. Reinfection tuberculosis—moderately advanced; bilateral	Slight	236	74	162
C.W. Reinfection tuberculosis—moderately advanced; progressive; bilateral	”	270	93	177
T.B. Reinfection tuberculosis—moderately advanced; cavity; bilateral	Moderate	232	71	161
L.V. Reinfection tuberculosis—far advanced; cavity closed later	Slight	181	74	107
H.P. Reinfection tuberculosis—far advanced since 1932	”	214	78	136
J.F. Reinfection tuberculosis—far advanced; bilateral	”	243	80	163
W.J. ” ” ” ” ”	Moderate	237	64	173
R.B. ” ” ” ” ”	”	284	90	194
A.H. ” ” ” ” ” progressive; died in 3 mo.	Severe	293	106	187
J.N. Reinfection tuberculosis—far advanced; bilateral; progressive; died next day	Moderate	307	110	197
S.H. Reinfection tuberculosis—far advanced; bilateral; progressive; died in 9 days	”	334	123	211

Note—Statistical analyses show the differences of the polysaccharide results from the normal to be highly significant.

the albumin peak. Evidence of similar heterogeneity has been noted in many of the sera from patients with far advanced tuberculosis but not in normal sera. Blix, Tiselius and Svensson[8] mentioned a similar experience with pneumonia serum, and found that the faster fraction contained the larger percentage of carbohydrate.

It is of interest that the polysaccharide found in tubercle bacilli or tuberculin, which has been shown by Renfrew,[10] Dorset and

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[10] Renfrew, A., *J. Biol. Chem.*, 1930, **89**, 619.

Henley,[11] Chargaff and Anderson,[12] and Heidelberger and Menzel[13] to consist of mannose, arabinose and galactose, gives a yellowish pink color[14] with the carbazole reagent, whereas glucose gives a bright pink color. The polysaccharide which can be detected in these serum protein fractions gives a similar yellowish pink color[†] and it has been shown by Rimington,[15] Bierry[16] and Hewitt[17] that the carbohydrate complex which they have found in serum proteins consists of mannose, galactose and glucosamine. Because of the similarity in composition in these two polysaccharides and in the color produced with the carbazole reagent, it was justifiable to use the standard tuberculin polysaccharide curve for translating the colorimeter readings into milligrams of polysaccharide. It is interesting to speculate whether the polysaccharide, found associated with the serum proteins and which increases during tuberculosis, is a normal constituent of the body, or whether it may be a foreign product, possibly produced by a pathogenic organism such as the tubercle bacillus. An answer might be reached by the application of a micro quantitative test for arabinose, which is a characteristic constituent of the tubercle bacillus polysaccharide.

Because of the suggestive nature of the results obtained it appeared worthwhile to determine in a simple way whether or not the polysaccharide content of whole serum increases in tuberculosis. Accordingly, total polysaccharide was determined on whole serum by applying the carbazole reaction[5] directly. Since the total carbohydrate of serum would include free glucose, the latter was determined on a second aliquot and the result subtracted from the total polysaccharide, giving the true polysaccharide content. In these studies the Shaffer-Hartmann reagent was used for determining the glucose, but any other approved method for glucose could be used instead. The purpose of these analyses was comparative rather than the determination of absolute values. Duplicate determinations could be run on a total of 2 ml of whole serum.

Analyses made in this manner on the sera of a series[‡] of normal, minimal, moderately advanced, far-advanced and terminal tuberculous cases, show (Table II) that the true polysaccharide content of serum does increase during tuberculosis, and to some extent proportionate to the extent of the disease. The increase could not be correlated with an increase in total protein content of the serum.

Correlation of the polysaccharide values with the clinical course of the disease has in many cases been highly suggestive. For example, J. H. in the "moderately advanced" group showed considerable clearing of the lesion on X-ray and a low value for polysaccharide, whereas C. W. showed progression in the lesion and a high polysaccharide value. L. V. in the "far advanced" group also showed great improvement, with closure of the cavity, and the polysaccharide value was practically normal, whereas several others who showed progression of disease and soon died from tuberculosis had extremely high polysaccharide values. While the average polysaccharide value among the "minimal" cases is higher than among the "normals," the values vary from a high normal to those found among the "far advanced" and some of the latter give the impression of clinical danger. However, the suggestive prognostic value of the test can not be established until many such cases are followed over a sufficient period of time in order to determine the actual course of the disease.

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[11] Dorset, M., and Henley, R. R., *J. Am. Vet. Med. Assn.*, 1930, **29**, 696.

[12] Chargaff, E., and Anderson, R. J., *Z. physiol. Chem.*, 1930, **191**, 172.

[13] Heidelberger, M., and Menzel, D. O., *J. Biol. Chem.*, 1937, **118**, 79.

[14] Seibert, F. B., Pedersen, K. O., and Tiselius, A., *J. Exp. Med.*, 1938, **68**, 413.

[†] Probably due to mannose which gives a similar color.

[15] Rimington, C., *Biochem. J.*, 1931, **25**, 1062.

[16] Bierry, H., *Compt. rend. soc. biol.*, 1928, **99**, 1837.

[17] Hewitt, L. F., *Biochem. J.*, 1936, **30**, 2229.

[‡] We express our appreciation to Dr. Horace R. Getz for these sera and discussion of the diagnoses.

14096

Secretion of Radio-active Sodium in Human Milk.

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The use of artificially activated substances provides an accurate method whereby labeled particles can be traced throughout the body. The passage of the isotope of sodium across the placenta has been described by Flexner and co-workers.[1,2,3] The authors[4] have recently been able to recover radio-active iron in the fetal cord plasma and fetal tissues within less than one hour after it had been fed the mother.[5]

Clinically there is no doubt that the suckling child may in varying degree be physiologically influenced by foods given the mother. Perlman and Dannenberg[6] have recently reported that nicotine is excreted in the milk and urine of all the mothers who smoked cigarettes. By

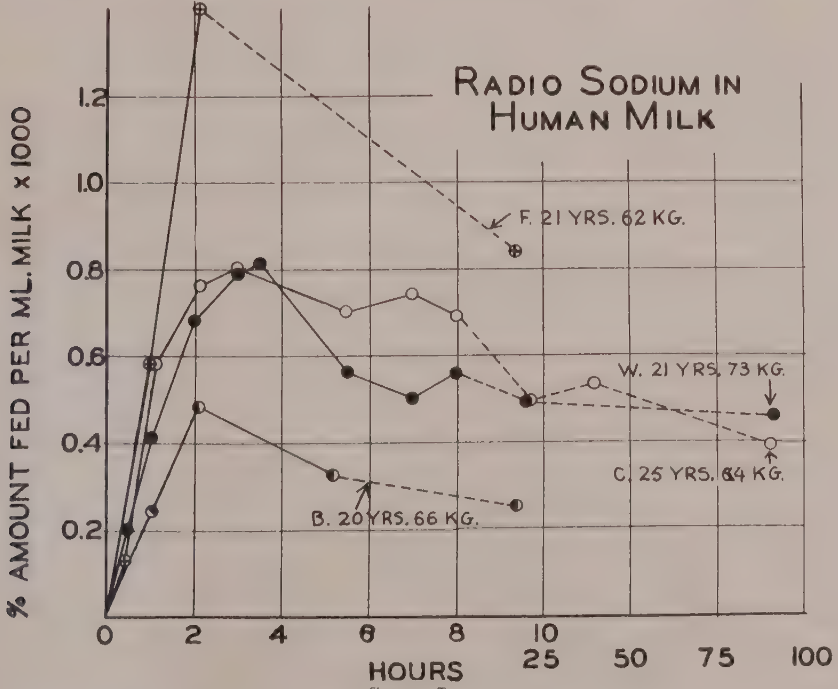

Chart I.

Showing values of radio-sodium output expressed as per cent of the amount ingested per liter of the mother's milk, plotted against time following ingestion.

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[1] Flexner, L. B., and Pohl, H. A., *J. Cell. Physiol.*, 1941, **18**, 49.

[2] Pohl, H. A., Flexner, L. B., and Gellhorn, E., *Am. J. Physiol.*, 1941, **134**, 338.

[3] Flexner, L. B., and Pohl, H. A., *Am. J. Physiol.*, 1941, **134**, 344.

[4] Pommerenke, W. T., Hahn, P. F., Bale, W. F., and Balfour, W. M., *Am. J. Physiol.*, 1942, **137**, 164.

[5] Unpublished data of authors.

[6] Perlman, H. H., and Dannenberg, A. M., *J.A.M.A.*, 1942, **120**, 1003.

the use of radio-active sodium* the authors have determined the speed with which a substance given by mouth to woman may be recovered in her milk.

Method. Four normal young women in the early postpartum state and varying in age from 20 to 25 years, and in weight from 62 to 73 kg, served as subjects. Only one breast of each subject was employed in this study, the other breast being used to nurse the baby. At the beginning of each experiment the selected breast was emptied with an electric pump. The subject was then given a single dose of about 65 mg of the radio-active sodium, as the chloride, in orange juice. A routine house diet was given, but no other drug was administered throughout the experiment. The breast was then emptied by means of a pump at frequent intervals so that the milk secretion for any particular period of time could be determined. Determination of radio-activity was made directly upon 2 ml of milk with a dipping type of tube[7] in conjunction with a Geiger-Muller counter. An aliquot of the radio-sodium fed was reserved in each case as a pilot sample and its activity was determined at frequent intervals. The resultant decay curve was used to establish the purity of the isotope preparation as well as to refer the individual activity measurements of milk to a convenient empirical zero time.

Results. In Chart 1 are shown the various values of radio-sodium output in the 4 cases expressed as percent of the amount ingested by the mother appearing in 1.0 liter of milk plotted against time following ingestion.

It is apparent from the results, as shown in Chart 1, that radio-sodium must be very rapidly assimilated since appreciable amounts of this substance may be recovered in the breast milk within 20 minutes after ingestion. The concentration of the isotope in the milk reaches a peak in about 2 hours, after which it drops off somewhat; and in about 6 to 8 hours it levels off to a fairly constant value. In 2 instances in which the concentration of the sodium was followed for as long periods as the half life of the isotope permitted, the concentration in the milk was about the same at 96 hours as it was at 20 hours.

In 2 of the cases the level of the radio sodium in the plasma was determined simultaneously with that of the milk on 2 occasions, *i.e.,* 16 and 66 hours respectively after ingestion. The ratios of plasma to milk labeled sodium can be seen in Table I.

TABLE I.

Subject	Time after ingestion, hr	Plasma sodium / Milk sodium
F	16	7.5
F	66	5.7
B	16	18.0
B	66	7.7

Summary. Radio-active sodium may be recovered in milk within 20 minutes after its ingestion. Its peak concentration in the milk is reached in about 2 hours. Subsequent secretion slowly tapers off, some of the sodium being recoverable after 96 hours.

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* The authors wish to express their appreciation to Dr. Gerhard Dessauer of the Department of Physics of the University of Rochester for furnishing the radio-active sodium used in this study.

[7] Bale, W. F., Haven, F. L., and LeFevre, M. L., *Rev. Scient. Instruments,* 1939, **10**, 193.

14097

Sulfanilamide Activity Against *Escherichia coli* Under Anaerobic Conditions.*

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The efficacy of sulfanilamide (SA) as an inhibitor of the aerobic growth of *Escherichia coli* in synthetic media and of *p*-aminobenzoic acid (PAB) as an anti-SA has been well established.[1,2,3] The studies of Broh-Kahn[4] and of Fox[5] raise some doubt as to the bacteriostatic activity of SA against *Esch. coli* under anaerobic conditions. The present study was carried out in an attempt to obtain more quantitative data on SA activity under anaerobic conditions of growth in a synthetic medium commonly employed in studies under aerobic conditions.

It was found (unpublished results) that high concentrations of SA markedly inhibit oxygen uptake of washed suspensions of *Esch. coli* (K-12) in the presence of sugars, amino acids, fatty acids, succinate, fumarate, lactate and pyruvate, the degree of inhibition depending to a considerable extent on the concentration of SA and of bacteria. PAB has little demonstrable anti-SA activity under these conditions and in relatively high concentrations by itself exerts an inhibitory action on respiration. PAB does effectively block SA inhibition of the aerobic growth and respiration of this strain of *Esch. coli* in the synthetic medium. SA has no appreciable effect on dehydrogenase activity with methylene blue as the hydrogen acceptor, even in saturated solutions of SA and after 2 to 5 hours' exposure of the organisms to the drug. Thus the inhibition of respiration produced by SA must occur after the initial activation of the substrate. If SA is an active depressant of respiration under anaerobic conditions, its point of attack may lie between the original dehydrogenase system and the final H-acceptor.

Experimental. The basal medium (Wood[3]) was diluted with an equal volume of sterile M/7.5 phosphate buffer of pH 7.2 (or a solution of Na bicarbonate) and inoculated with a previously centrifuged suspension of an 18-hour thioglycollate broth culture. Essentially similar results were obtained with inocula from cultures in the synthetic medium with the exception that a longer lag period of growth was observed. The inoculated medium was distributed in vented Warburg flasks containing measured volumes of SA and PAB solutions and the air in the flasks immediately displaced with O_2-free H_2 or N_2 or with a 95% H_2-5% CO_2 mixture when bicarbonate was employed as the buffer system. In a number of duplicate experiments a small particle of phosphorus was present in each flask to ensure complete removal of oxygen. However, no difference in results was observed in the presence or absence of phosphorus.

Attempts were made to determine the amounts of CO_2 and of H_2 produced during growth. However, the reduced CO_2-tension produced with KOH as the CO_2-absorbent markedly increases the lag period of growth of *Esch. coli* under anaerobic conditions, the amount of growth in 6 hours generally being 50 to 75% less than in the absence of KOH. The manometric results will therefore be reported as CO_2. Growth was estimated from turbidimetric measurements in a Klett-Summerson photoelectric colorimeter. All experiments were carried out at 37°C.

Results. A comparison of the activity of SA under aerobic and anaerobic conditions is presented in Table I. A heavier inoculum (3x) was employed in the anaerobic culture and growth was allowed to proceed 90 minutes longer.

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* Aided by a grant from the Fluid Research Fund of Stanford University School of Medicine.

[1] Kohn, H. I., and Harris, J. S., *J. Pharm.*, 1941, **73**, 383.

[2] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

[3] Wood, W. B., Jr., *J. Exp. Med.*, 1942, **75**, 369.

[4] Broh-Kahn, R. H., *Science*, 1939, **90**, 543.

[5] Fox, C. L., Jr., *Science*, 1940, **91**, 477.

TABLE I.

Influence of SA on Aerobic and Anaerobic (in H_2) Growth and Respiration of *Esch. coli.*

		Mm³ gas				
		O_2	as CO_2	% control	Bact./ml $\times 10^8$	% control
Control	Aerobic	161			19.8	
	Anaerobic		168		19.5	
SA M/12,800	Aerobic	50		31.1	5.0	25.3
	Anaerobic		62	36.9	13.5	69.2
SA M/25,600	Aerobic	69		42.9	9.0	45.0
	Anaerobic		118	70.3	16.5	84.6
SA M/51,200	Aerobic	95		59.0	11.5	58.1
	Anaerobic		136	80.9	19.5	100

TABLE II.

Influence of M/1600 SA on Anaerobic (in N_2) Respiration and Growth of *Esch. coli* per ml of Synthetic Medium.

	No. bact.	Total Mm³ CO_2	Mm³ CO_2/cell/hr
Control			
0 hr	2.42×10^8	—	—
2.5 "	3.85 "	20	2.6×10^{-8}
4.5 "	16.83 "	132	6.3 "
5.5 "	34.10 "	545	16.9 "
+ SA M/1600			
0 hr	2.42×10^8	—	—
2.5 "	3.85 "	18	2.4×10^{-8}
4.5 "	6.82 "	57	3.8 "
5.5 "	10.12 "	101	5.2 "
% of control	29.7	18.5	

Table I shows that, under our experimental conditions, SA inhibits respiration and growth to a similar extent under aerobic or anaerobic conditions. With the heavier inocula necessitated by the slower rate of growth under anaerobic conditions, it is necessary to use somewhat higher concentrations of SA than under aerobic conditions. SA also exerts its inhibitory action on growth in the bicarbonate buffered as well as in the phosphate buffered medium. In the bicarbonate medium CO_2 production appears to be inhibited to a greater extent than acid production. It has been observed (Sevag,[6] unpublished results) that the carboxylase system is quite sensitive to the sulfa-drugs. However, the studies on SA activity in the bicarbonate medium are complicated by the marked changes observed in pH due to acid production with consequent decomposition of the bicarbonate. SA activity is of the same magnitude in an atmosphere of H_2 as of N_2 with the possible exception that H_2 itself may exert a slight inhibitory action on gas production (Woods[7]).

In Table II results are presented showing the influence of SA on growth and respiration during the early stages of growth in the synthetic medium under anaerobic conditions.

It is apparent that the rates of respiration and of growth are inhibited by SA to a similar extent during anaerobic growth in the synthetic medium. Also, a lag period in SA inhibition is observed under anaerobic conditions similar to that usually observed in aerobic cultures. It must be borne in mind that this lag period of SA inhibition of respiration is not observed with washed suspensions.

The inhibitory effect of SA can be prevented by the addition of PAB to the culture medium.

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[6] Sevag, M. G., Shelburne, M., and Mudd, S., *J. Gen. Physiol.*, 1942, **25**, 805.

[7] Woods, D. D., *Biochem. J.*, 1936, **30**, 515.

In a typical experiment 17 mm^3 of gas was produced in 7.5 hours in the presence of M/1600 SA as compared with 190 in the control and 172 in M/1600 SA + M/200,000 PAB. The corresponding growth figures are 5.2, 25.3 and 25.4 bacteria × 10^8 per ml respectively.

Conclusions. The results of this study show that SA inhibits the respiration and growth of *Esch. coli* in a synthetic medium with glucose as the source of energy under either aerobic or anaerobic conditions and that PAB also exerts its anti-SA activity under anaerobic conditions. Growth and respiration are inhibited to somewhat the same extent as previously reported by Sevag[8] for *Streptococcus pyogenes* and Type I *Diplococcus pneumoniæ*. SA appears to inhibit respiration somewhere between the original dehydrogenase system and the final H-acceptor, possibly inhibiting a H-carrier system as well as the decarboxylase system. It is not evident whether the decreased rate of respiration is due to inhibition of growth or vice versa although Wyss[9] reports that respiration is inhibited by isomers of SA which do not exhibit "chemotherapeutic activity."

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[8] Sevag, M. G., and Shelburne, M., *J. Bact.*, 1942, **43**, 447.

[9] Wyss, O., Strandskov, F. B., and Schmelkes, F. C., *Science*, 1942, **96**, 236.

14098

Alteration of Crystalline Androsterone and Dehydroisoandrosterone by Alcohol as a Vehicle.

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Investigations were undertaken to evaluate the respective merits of alcohol and oil as vehicles for androgenic assays after having observed[1] that the degree of response of the chick's comb to androsterone, dehydroisoandrosterone,* and varying mixtures of these compounds was modified by the vehicle in which they were dissolved. Klempner, Frank and Hollander[2] noted a greater increase in the weight of chicks' combs treated with crystalline androsterone dissolved in 95% alcohol over that resulting from the use of an oily solution. They consequently recommended alcohol as the vehicle of choice. The enhancement of tissue response was ascribed by them to either better absorption of the hormone from the alcoholic solution or to an increased rate of evaporation of the vehicle. Our results indicate that changes occur in such alcoholic solutions, making this vehicle unsatisfactory for biological assays of these androgens.

Experimental. In these investigations we employed the chick-comb-weight method, as previously described.[3] Although several workers[4,5,6] have questioned the value of the chick as a test animal, our extensive experience during the past 4 years, with routine and experimental androgenic assays, has shown that with careful technic, standard conditions as to light and temperature, and sufficiently large groups of animals, the chick's comb will give a consistent response well within the accepted limits of biological error.

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[1] Grauer, R. C., Starkey, W. F., and Saier, E., studies in progress.

* The androsterone and dehydroisoandrosterone used in these studies were kindly supplied by the Ciba Pharmaceutical Products through the courtesy of Dr. Ernst Oppenheimer.

[2] Klempner, E., Frank, R. T., and Hollander, F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 633.

[3] Starkey, W. F., Grauer, R. C., and Saier, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 649.

[4] Duff, P. A., and Darby, H. H., *Endocrinology*, 1941, **28**, 643.

[5] McCullagh, D. R., and Guillet, R., *Endocrinology*, 1941, **28**, 648.

[6] Hoskins, W. H., Beach, G. W., Coffman, J. R., and Koch, F. C., *Endocrinology*, 1941, **28**, 651.

A total of 2,151 unsexed chicks was used in this study, 105 being controls treated with the vehicle free from hormone. A constant amount of 0.01 cc of the solution containing the androgen (1 to 40 γ doses) was applied to the combs of 2-day-old chicks for 6 consecutive days, in accordance with our previous report.[3] Groups of 12 to 32 animals were used at a given dose and the response was expressed as a comb-weight (mg)/body-weight (g) ratio. From these data, suitable curves were calculated by the method of least squares. This study was primarily concerned with the use of alcohol as a vehicle; the results obtained with the compounds dissolved in oil were reviewed from a part of another study, for the purpose of comparison. These investigations were designed to determine if alcohol produced a change in the dissolved androgens, thus enhancing their activity. Two methods were employed using alcohol as the vehicle; a "standing" and an "immediate" method. The "standing method" of treatment consisted of making up the alcoholic solutions on the first day and using them throughout the 6 days of the experiment. The solutions were kept tightly stoppered and under refrigeration when not in use. The "immediate method" consisted of dissolving the crystalline hormone in 95% alcohol and applying the solution to the chicks' combs immediately. Five minutes was the maximum elapsed time from the beginning of the procedure to completion of the application of each "run." Fresh solutions were made daily.

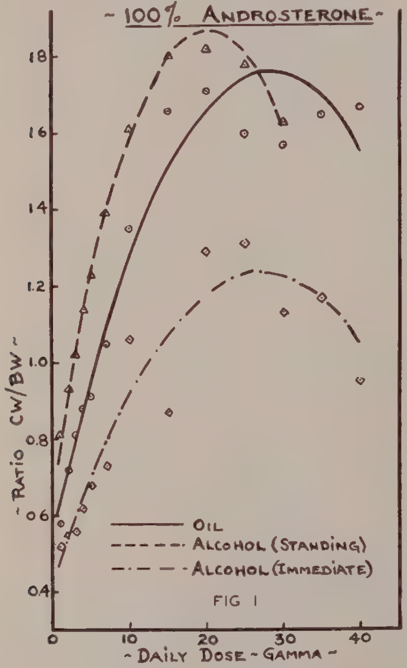

FIG 1

In order to determine whether prolonged contact of the androgens with alcohol resulted in some alteration of the compounds, crystalline androsterone and dehydroisoandrosterone were dissolved in 95% alcohol (USP) and the solutions allowed to stand for 6 days. The alcohol was then evaporated at room temperature, the crystals recovered, and dissolved in sesame oil. These oily solutions were tested for androgenic activity by the chick-comb, weight method and the curves plotted in the manner previously described.

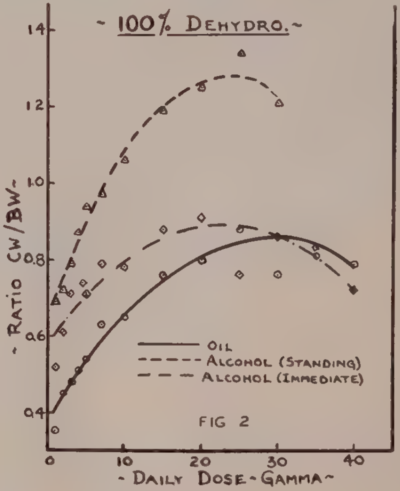

FIG 2

Results. With all solutions the curves were parabolic in type. The "standing method" gave a greater response than did either the "immediate method" or the oily solutions. The

25% androsterone + 75% dehydroisoandrosterone mixture (Fig. 4) showed the greatest difference between the two methods. This mixture also gave the least response when the "immediate method" was employed. The oily solutions gave greater responses than did the "immediate method" (Fig. 1, 3, 4) except in the case of the 100% dehydroisoandrosterone dissolved in alcohol which, by the "immediate method," was slightly superior to the oily solution (Fig. 2). The greatest difference in response between alcoholic (standing method) and oily solutions was noted with 100% dehydroisoandrosterone (Fig. 2).

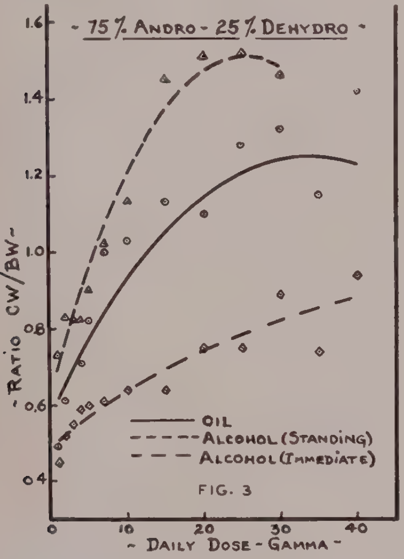

FIG. 3

The androsterone recovered after alcohol treatment for 6 days gave a much lower response than did the original material (Fig. 5). The original compound reached an optimum response at the 25-30 γ daily dose, whereas with the alcohol treated androsterone the optimum was not reached even at the 40 γ dose. The alcohol treated dehydroisoandrosterone (Fig. 6) was seen to be a more potent androgen than the original compound dissolved in the inert sesame oil. The type of response is similar to that of alcohol treated androsterone (Fig. 5), the slope of the curve continuing upward even at the 40 γ dose.

Discussion. The increasing use of alcohol as a vehicle for androgenic material warrants a note of caution. We observed[1] that varying mixtures of the biologically active androgens, androsterone and dehydroisoandrosterone, due to synergistic action, yield varying results insofar as the response of the chick's comb is concerned. From our present report, it becomes apparent that the vehicle in which these androgens are dissolved may be an additional factor in determining the nature of the response. It is therefore important for us to minimize the number of these variable factors and consequently suggest that an inert vehicle such as oil is to be preferred.

Previous workers[2,7,8,9,10] have noted that androgens dissolved in alcohol gave a greater biological response than when in oily solution, but none of them has stated the length of time the androgens were in contact with the alcohol. The possibility that a change occurred in the compounds as a result of their prolonged

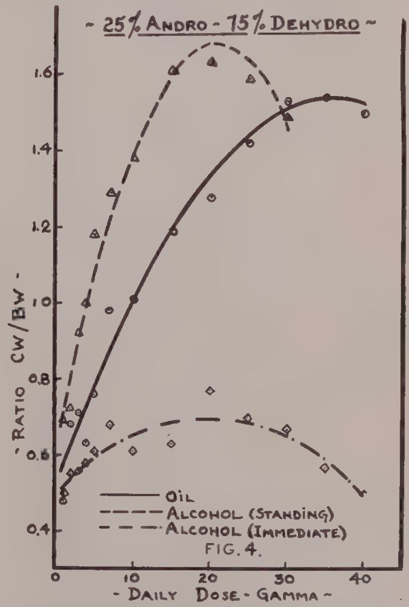

FIG. 4.

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[7] Koch, F. C., *The Harvey Lectures*, 1937-38.

[8] Zondek, B., and Sulman, F., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 633.

[9] Greenwood, A. W., Blyth, J. S. S., and Callow, R. K., *Biochem. J.*, 1935, **29**, 1400.

[10] Callow, R. K., and Deanesley, R., *Biochem. J.*, 1935, **29**, 1425.

contact with alcohol should be considered. While we have no absolute proof of chemical or physical alteration of the original androsterone and dehydroisoandrosterone due to prolonged contact with alcohol, the quantitative biological response and the type of response curve obtained are suggestive of a difference in these compounds before and after alcohol treatment. Lack of an enhanced response by the "immediate method" of treatment indicated that the production of a more biologically active substance does not occur rapidly. If the original and alcohol treated products were the same, the response curves for both preparations should be identical when an inert vehicle such as sesame oil is employed. This, however, is not the case.

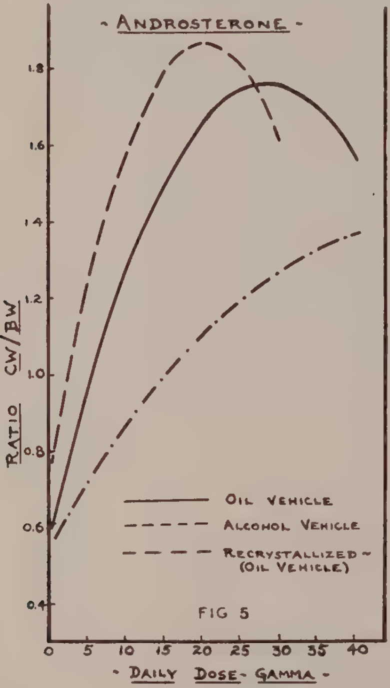

FIG 5

Summary. Prolonged contact of crystalline androsterone and dehydroisoandrosterone with alcohol results in an enhanced biological response as compared with the response obtained from oily solutions of these androgens, when tested by the chick-comb-weight method. When these androgens are applied to the combs immediately after going into alcoholic solution, such an enhancement does not occur.

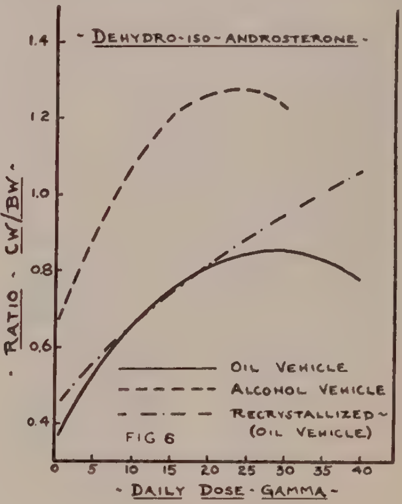

FIG 6

Recovery of the crystals from alcoholic solution, after 6 days contact, and subsequent solution in oil gives an altered biological response indicating a possible change in the character of the original materials. These observations suggest that an inert vehicle, such as sesame oil, is to be preferred to alcohol as a vehicle in assaying such androgens.

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Effects of Prolactin and Cortical Hormones on Body Weight and Food Intake of Adrenalectomized Pigeons.

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Earlier publications[1,2] have shown that in pigeons both prolactin and adrenal cortical hormones can prevent or reduce the loss in weight of body and viscera which otherwise occurs during the 10 days immediately following hypophysectomy. The clear demonstration of this action of cortical hormones made it necessary to learn whether the similar action of prolactin on body weight and food intake is mediated by the adrenals. Although it was known that most of the preparations of prolactin used in these particular studies[1,2] were almost free of any ability to enlarge the adrenals of 2-day chicks and 21-day rats only tests of effects of prolactin in completely adrenalectomized pigeons could resolve this question. Since cortical hormone preparations had shown additive effects with prolactin on body weight and food intake of hypophysectomized pigeons[2] two cortical preparations were included in the present study. War conditions have made it necessary to discontinue this work before all contemplated tests could be made, but the results already obtained seem to warrant publication.

Methods. Carneau pigeons aged 1.5 months from hatching were adrenalectomized in 2 stages under nembutal anesthesia with an interval of one week between the operations. The technic is essentially similar to that independently developed by Bülbring.[3] Each adrenal is removed in one piece by blunt dissection. At death or at killing the completeness of removal was determined by histological examination of any doubtful tissue. Incomplete removal of the adrenals was relatively rare and data from such animals are not included here. Immediately before the second operation 1 mg of desoxycorticosterone acetate was injected. During experimental periods salt (NaCl, .75 g, $NaHCO_3$, .125 g) in gelatin capsules was given twice daily unless the birds were injected with a cortical hormone.

All injections were begun on the day of the second operation. Adrenal cortex extract* (ACE) was given twice daily and desoxycorticosterone acetate† (DCA) or prolactin once daily. The preparation of prolactin used contained, according to our methods of assay,[4] the following units per mg of hormones: prolactin, 17; gonadotrophin, $<.004$; thyrotrophin, 0.06; corticotrophin, .024. The total amount of food and water consumed in each period was measured and the average daily intake per bird calculated.

Results. When given salt as noted above adrenalectomized young Carneau pigeons have an average survival period of 9 days after removal of the second adrenal; none has lived longer than 17 days without cortical hormone therapy. Drakes are reported[3] to survive 8 to 10 hours, whereas fowl are said[5] to live for as long as 82 days after adrenalectomy. Adrenalectomized pigeons lose weight slowly at first and then rapidly during a day or two before death (Fig. 1). Daily food intake in the 10-day period after operation is reduced

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[1] Schooley, J. P., Riddle, O., and Bates, R. W., *Am. J. Anat.*, 1941, **69**, 123.

[2] Miller, R. A., and Riddle, O., Abst. in *Anat. Rec. Suppl.*, 1942, **84**, 40; complete paper, *Endocrinology*, 1943, in press.

[3] Bülbring, E., *J. Physiol.*, 1937, **89**, 64.

* Adrenal cortex extract was supplied through the courtesy of Dr. David Klein, The Wilson Laboratories.

† Desoxycorticosterone acetate was supplied through the courtesy of Dr. Erwin Schwenk, Schering Corporation.

[4] Bates, R. W., Riddle, O., and Miller, R. A., *Endocrinology*, 1940, **27**, 603.

[5] Herrick, E. H., and Torstveit, O., *Endocrinology*, 1938, **22**, 469.

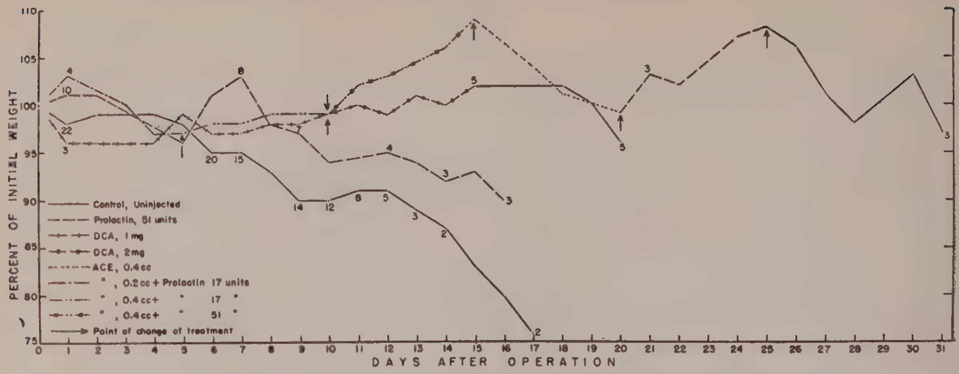

FIG. 1.

Changes in body weight of adrenalectomized pigeons injected with cortical hormones and prolactin. The figures on each curve indicate the number of surviving animals.

to about one-fifth the amount eaten by normal unoperated pigeons (Table I). Water intake was apparently unaffected by adrenalectomy but it was increased markedly when salts were given.

The effects of prolactin, DCA and ACE on body weight and on food and water consumption are presented in Table I and Fig. 1. One mg daily of DCA partially supported food consumption for two 5-day periods but did not cause an increase in body weight; 2 mg restored growth rate and food intake to levels characteristic of normal unoperated pigeons. Water intake was not appreciably changed by DCA. It is notable, however, that similar amounts of DCA markedly stimulated the water consumption of hypophysectomized pigeons.[2] When no treatment was given during a final 5-day period (15-20 days) following 2 mg DCA daily body weight remained constant for a few days and then declined. In this case prolonged absorption of DCA from the sesame oil solution injected during the preceding 15 days probably accounts for the temporary maintenance of body weight and a level of food intake somewhat higher (Fig. 1) than that found in other untreated adrenalectomized pigeons.

Prolactin caused an increase in the weight of adrenalectomized pigeons when adrenal insufficiency was not acute. All of the pigeons of Group III regained at least some of the weight lost after adrenalectomy and most of them became considerably heavier than at operation; all of them, however, lost weight sooner or later as adrenal insufficiency developed even though prolactin was being administered. In the averages presented for birds of Group III (Table I, Fig. 1) the marked gains in weight by some pigeons are largely concealed by the heavy losses in other moribund animals. The ability of prolactin to stimulate an increase in the body weight of adrenalectomized pigeons was again clearly demonstrated by the animals of Group V (Fig. 1) during the fifth period (20-25 days) after operation. These pigeons had been maintained previously with adrenal cortical extract. It seems probable that the loss of weight during the immediately preceding period (15-20 days) was not the result of insufficient dosage with ACE but the result of discontinuing an earlier series of injections of prolactin (Table I, Fig. 1).

Either prolactin or ACE partially maintained food consumption, and this partial support of appetite by each hormone appears to be additive when both substances were injected into the same adrenalectomized pigeons (Table I). When amounts of ACE similar to those given here were injected into hypophysectomized pigeons water intake was greatly stimulated.[2]

Discussion. The above results, and the literature cited, demonstrate that prolactin stimulates more than normal increases in the weight of pigeons in the absence of either the adrenals or the anterior pituitary, while cortical hormones restore a normal rate of growth in adrenalectomized pigeons but only partially

TABLE I.
Effects of Adrenal Cortical Hormones and Prolactin on Food and Water Intake and on Body Weight of Adrenalectomized Young Carneau Pigeons.

				Data obtained end of each period		Intake	
Group	Substance injected	Daily dose	Days after operation	Wt change, %	No. of birds	Food per day, g	Water per day, cc
I	Normal, uninj.	—	1-10	+ 5	15	31	56
		Adrenalectomized.					
II	Uninj.*	—	1-10	—10	12	7	120
III	Prolactin*	51 u	1- 5	— 4	10	19	139
	,,	51 u	5-10	— 3	7	16	140
	,,	51 u	10-16	— 4	3	23	93
IV	DCA	1 mg	1- 5	— 1	3	18	77
	,,	1 mg	5-10	— 0	3	19	52
	,,	2 mg	10-15	+ 3	3	39	60
	Uninj.	—	15-20	— 6	3	22	58
V	Prolactin + ACE	17 u 0.2 cc	1- 5	— 3	4	22	56
	Prolactin + ACE	17 u 0.4 cc	5-10	+ 2	4	26	58
	Prolactin + ACE	51 u 0.4 cc	10-15	+10	4	44	70
	ACE	0.4 cc	15-20	—10	4	26	57
	Prolactin*	51 u	20-25	+ 9	3	28	111
	Uninj.*	—	25-31	—11	3	8	97

*Salt mixture given by mouth twice daily (total, 1.5 g NaCl; 0.25 g $NaHCO_3$).

support the weight of hypophysectomized pigeons. It is evident therefore that prolactin is of primary importance in this aspect of growth in pigeons and that this action of prolactin is not mediated through the adrenal. One of us[6] earlier reported some data which indicate that the ability of prolactin to increase the basal heat production of pigeons is also not mediated by the adrenals. In adrenalectomized rats Long[7] observed that although anterior pituitary extract caused a gain in weight (rats maintained on salt), still better responses were always observed if the animals received cortical extract in addition. It should be remembered, however, that for pigeons the main growth promoting pituitary factor is prolactin and for the rat it is some other pituitary factor or a combination of factors.

Summary. Adrenalectomized young Carneau pigeons fed 1.75 g of a salt mixture daily showed an average period of survival of 9 days. Such pigeons eat little food and lose weight. Prolactin induced more than normal gains in body weight when adrenal insufficiency was not acute and restored food intake to a level almost equal to that of unoperated birds. Either adrenal cortical extract or desoxycorticosterone acetate maintained life in adrenalectomized pigeons. Daily doses of 2 mg of desoxycorticosterone acetate fully restored the rate of increase of body weight and the daily food consumption to levels characteristic of normal unoperated pigeons. A group of birds given high dosage of both prolactin and adrenal cortical extract showed more rapid gains in body weight and larger food intake than has ever been observed in normal unoperated pigeons of the age used here.

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[6] Riddle, O., and Smith, G. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 499.

[7] Long, C. N. H., *Cold Spring Harbor Symposia*, 1942, **10**, 91.

14100

Influence of Thyroactive Iodocasein on Growth of Chicks.

Jesse E. Parker. (Introduced by C. W. Turner.)

From the Department of Poultry Husbandry, University of Tennessee Agricultural Experiment Station, Knoxville, Tenn.

Koger, Hurst and Turner[1] have reported that thyroxin has a stimulating effect on the rate of growth of female albino mice; and more recently[2] that thyroactive iodocasein has a similar growth-stimulating property. This report presents data relative to the effect of such material* on body and feather growth and on the efficiency of feed utilization of chicks to 12 weeks of age.

Methods. Five lots of Rhode Island Red chicks were started in a battery brooder on May 21, 1942, and when 8 weeks old were transferred to a finishing battery. Thyroactive iodocasein was added to the basal all-mash ration of 4 groups at levels varying from 0.025% to 0.2% One group, the controls, received the basal mash only. All chicks were weighed individually in grams at 1 day and at 4, 8 and 12 weeks of age. The room in which the experiments were conducted was not air-conditioned, and the relatively high prevailing summer temperatures undoubtedly had a suppressing influence on the rate of growth of all groups. At 12 weeks the chicks were examined as to feather growth and placed in 3 grades, as follows: *Rapid*—Chicks well covered with feathers; *medium*—chicks fairly well covered, but not completely covered over backs and wing fronts, and with few pin feathers; and *slow*—poorly feathered chicks, including "bare-backs" and those with many immature or "green" feathers.

Results. The results presented in Table I indicate that thyroactive iodocasein has a growth-stimulating effect on chicks to 12 weeks of age. At 4 weeks of age the mean weights of the 5 groups were about the same, but at 8 weeks the thyroactive iodocasein-fed chicks were consistently heavier. The chicks receiving the mash with the highest level of thyroactive iodocasein (0.2%) averaged 12% heavier than the controls. At 12 weeks, however, the chicks on the lowest level of thyroactive iodocasein (0.025%) weighed the most. The average gains made by the 5 groups from 4 to 8 and 8 to 12 weeks and from 1 day to 12 weeks are shown in Table II. The fact that the chicks in Lot 5 made the greatest gains from 4 to 8 weeks, whereas those in Lot 2 made the greatest gains from 8 to 12 weeks is of interest, and suggests that the levels of thyroactive iodocasein conducive to greatest gains change with the age or stage of development of the chick.

Although the differences in the amounts of feed required per gram gain in the 5 groups were not great (Table II), there is some indication that the 2 groups receiving the smallest amounts of thyroactive iodocasein required less feed per unit gain than did the controls or the chicks fed the higher levels. The fact that Lot 5 required more feed per unit gain than did the controls, and Lot 4 as much as the controls, whereas the average gains of both lots exceeded those of the controls, indicates that feeding the higher levels of thyroactive iodocasein tends to decrease the efficiency of feed utilization. Koger, Hurst and Turner observed that thyroxin treatment of mice increases the efficiency of gains only during the time that the rate of growth is more rapid than in non-treated animals. The data on gains and feed utilization shown in Table II

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[1] Koger, M., Hurst, V., and Turner, C. W., *Endocrin.*, 1942, **31**, 237.

[2] Koger, M., Reineke, E. P., and Turner, C. W., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 236.

* The thyroactive iodocasein was furnished by Drs. C. W. Turner and E. P. Reineke, Department of Dairy Husbandry, University of Missouri, and was prepared by the method described by Reineke *et al.*, *J. Biol. Chem.*, 1942, **143**, 285. The preparation used in this experiment showed approximately 5.4% of the response of thyroxine when assayed by injection into tadpoles. This is equivalent to an oral effect 2% that of thyroxine.

TABLE I.
Influence of Thyroactive Iodocasein on Average Body Weights of Rhode Island Red Chicks to 12 Weeks of Age. Both Sexes.*

Lot No.	% of thyroactive iodocasein in ration, %	Chicks No.	Chicks Sex ♀	Age 1 day g	Age 4 wks g	Age 8 wks g	Age 12 wks g	12-wk wt to control wt, %	P† value, %
1	.000	23	13	41.8	141.1	423.1	789.4	100.0	
2	.025	23	14	41.4	139.5	447.7	861.5	109.1	5
3	.050	24	14	40.8	149.6	445.6	836.2	105.9	30
4	.100	23	12	42.2	142.9	445.9	819.0	103.7	60
5	.200	22	12	42.6	148.9	474.8	841.9	106.7	25

*Avg body weights for both sexes $= \dfrac{\text{Avg wt males} + \text{avg wt females}}{2}$

†Probability that the 12-wk wt of the group under consideration does not differ from that of controls in lot 1.

TABLE II.
Influence of Thyroactive Iodocasein on Gains and Efficiency of Feed Utilization of Rhode Island Red Chicks. Both Sexes.

Lot No.	% thyroactive iodocasein in ration, %	Gains and (amounts of feed per gram gain) 4-8 weeks g	g	8-12 weeks g	g	0-12 weeks g	g
1	.000	282.0	(3.55)	366.3	(4.14)	747.6	(3.90)
2	.025	308.2	(3.40)	413.8	(3.87)	820.1	(3.72)
3	.050	296.0	(3.50)	390.6	(4.05)	795.4	(3.76)
4	.100	303.3	(3.55)	373.1	(4.12)	776.8	(3.90)
5	.200	325.9	(3.48)	367.1	(4.57)	799.3	(4.05)

TABLE III.
Influence of Thyroactive Iodocasein on Feathering of Rhode Island Red Chicks.

		Rate of feathering									
		Cockerels			Pullets			Both sexes			
Lot No.	% thyroactive iodocasein in ration %	Rapid %	Medium %	Slow %	Rapid %	Medium %	Slow %	Rapid %	Medium %	Slow %	Chi-* Square
1	.000	0	40	60	15	69	15	9	56	35	7.19
2	.025	0	0	100	29	50	21	17	30	52	4.49
3	.050	0	10	90	7	64	29	4	42	54	9.90
4	.100	36	55	9	83	8	8	61	30	9	6.54
5	.200	90	10	0	92	8	0	91	9	0	27.68

*Total $X^2 = 55.50$, df $= 8$, $P = <1\%$. The sexes were not treated separately since numbers were too small for a valid Chi-Square test. From observation, it is evident that thyroactive casein in large dosages caused a marked rapid feathering in both sexes.

show that a similar phenomenon exists when thyroactive iodocasein is fed to growing chicks.

Data presented in Table III show that levels of 0.1 and 0.2% of thyroactive iodocasein in the chick ration stimulate feather growth. Feather development in the 2 groups of chicks receiving the lower levels, 0.025 and 0.050%, was no better than that of the chicks in the control group, if as good. From a practical standpoint it is unfortunate that the chicks which made the greatest and most economical gains did not feather more satisfactorily. It is possible that thyroactive iodocasein may be used to stimulate both body growth and feather growth by varying the amounts fed at different ages.

Conclusions. Rhode Island Red chicks were raised to 12 weeks of age on diets containing from 0.025 to 0.2% of thyroactive iodocasein. Chicks receiving the lower levels, 0.025 and 0.050%, made greater gains and required less feed per gram of gain than did the controls. Those fed diets with 0.1 and 0.2% thyroactive iodocasein gained more than the controls and were more fully feathered than either the controls or those receiving the lower levels. The thyroactive iodocasein-fed chicks utilized their feed more efficiently than the controls only during the periods when they were gaining at a more rapid rate.

Statistical analysis showed the difference in rate of gain to be of doubtful significance although the data indicate that at the proper dosage level growth rate was above normal. The differences in the rate of feathering in favor of the treated birds on higher dosage levels were highly significant.

14101

Influence on Growth of Thyroactive Iodocasein.*

MARVIN KOGER, E. P. REINEKE AND C. W. TURNER.

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We have shown recently that small amounts of synthetic thyroxine injected over a period of 5 weeks will cause an acceleration in the growth rate of immature mice.[1] With the discovery that a highly potent thyroactive iodoprotein[†] could be formed by combination of iodine with tyrosine-containing proteins under controlled conditions,[2,3] it was decided to test the effect of some of these products on the growth rate of mice and determine whether responses similar to these secured by giving small amounts of thyroxine would be obtained.

Materials and Methods. Virgin female mice weighing from 13 to 15 g at the beginning of the trials were used as experimental animals. Three different trials were conducted over a period of 35 days each, different lots of thyroactive iodocasein being used for each trial. Suitable controls were kept for each experiment. The animals were numbered individually and their lengths determined under anesthesia at the beginning and at the end of the experimental period. Body weights were taken weekly. The dosages and methods of administration of the thyroactive iodocaseins as well as the grouping of animals in the experiments are indicated in Table I.

The thyroactive proteins used were prepared by iodination of casein at the optimal level as described in previous reports.[2,3]

The material used in Experiment 1 was a highly potent preparation containing 7.14% iodine. It possessed 10.3% of the potency of synthetic thyroxine-sodium (BDH) on a weight basis when injected into tadpoles, and 4% of the activity of thyroxine when assayed on guinea pigs, by oral administration of the test substance. The material was injected subcutaneously at the rate of 0.55 mg every 2 days. This dosage was calculated purposely to contain the same amount of iodine as 0.06 mg synthetic thyroxine—an amount of thyroxine that has proven to cause acceleration of growth rate in young mice.[1]

The material used in Experiment 2 was a less potent lot containing 1.8% the activity

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[*] Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 846.

[†] The term thyroactive iodocasein is used to designate products formed by iodination of proteins in such a manner as to produce a substance with high thyroidal activity. It is necessary to distinguish between these preparations and compounds such as iodocasein and iodoalbumin which are used merely as carriers of organic iodine, and have little or no thyroidal effect.

[1] Koger, Marvin, Hurst, Victor, and Turner, C. W., *Endocrinology*, 1941, **29**, 667.

[2] Reineke, E. P., Williamson, M. B., and Turner, C. W., *J. Biol. Chem.*, 1942, **143**, 285; 1943, **147**, 115.

[3] Reineke, E. P., and Turner, C. W., *Mo. Agri. Exp. Sta. Bull.* 355, 1942.

TABLE I.
Effect of Thyroactive Iodocasein on Body Weight Gain and Gain in Skeletal Length During a 5-Week Period.

			Body wt				Nose-tail length			
				Diff. in gain (treated above controls)				Diff. (treated above controls)		
Exp. No.	Treatment	No. animals	Mean gain, g	g	%	P value,* %	Mean gain, %	mm	%	P value,* %
1	Controls	7	6.4				17.3			
	0.548 mg inj. alternate days	7	7.9	1.5	23	>5	25.3	8.0	46.2	<5
2	Controls	11	7.7				25.8			
	0.3 to 0.5% of ration	16	8.9	1.2	16	>5	30.9	5.1	19.8	<5
3	Controls	22	6.4				16.4			
	.04% of ration	24	7.5	1.1	17	5	20.6	4.2	25.6	<5
	.08% ” ”	22	7.4	1.0	16	5	20.7	4.3	26.2	<5
	.16% ” ”	20	7.6	1.2	19	5	21.8	5.4	32.9	<5
	.32% ” ”	11	7.9	1.5	23	5	20.9	4.5	27.4	5
Total	Controls	40								
	Treated	100		1.2	18	<1		4.9	27.9	<1

*Probability that the differences observed are due to random fluctuation.

of thyroxine when injected into tadpoles. The preparation was not assayed with guinea pigs. It was given in the feed at the level of 0.3% to 0.6% of the ration.

In Experiment 3 a potent preparation containing 7.24% iodine was used and fed the different groups of animals, in graded amounts of 0.04, 0.08, 0.16 and 0.32% of the ration. The material had 9.3% the potency of thyroxine as assayed by injection into tadpoles.

Results. A summary of the growth data obtained from the different trials is presented in Table I. Without exception, the treated animals gained more in body weight and length than their corresponding controls. The thyroactive iodocasein-fed animals gained from 16 to 23% more in weight and an average of 28% more in length than the untreated controls. In Experiment 3 where graded amounts of the thyroactive iodocasein were fed, there was a tendency for the gains to be greater in the groups receiving 0.16% and 0.32% than in the groups receiving 0.04% and 0.08% in the ration.

Analysis of variance[4] showed the difference in gains in body weight and in length in favor of the treated animals to be significant for the larger groups of animals in Experiment 3 ($P = 5\%$ or less). In the smaller groups in Experiment 1 and 2 the differences in body weight gain were not significant for the numbers of animals included. The differences were in favor of the treated animals, however, and the differences in gain in body length were significant. When the data were summated (see totals in Table I), the differences in gain in both weight and in skeletal growth (length) in favor of the treated animals were highly significant (probability value of less than 1%).

The differences in gain in skeletal growth (length) are somewhat more marked and are significant even when only small groups are compared.

Summary. Immature virgin female mice were treated with thyroactive iodocasein by both oral and subcutaneous administration and their growth compared to that of controls. The treated animals gained significantly more in body weight and in total length than the non-treated controls.

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[4] Snedecor, George W., *Statistical Methods*, Iowa State College Press, 1940.

14102

Production of Local Skin Reactivity by Passive Transfer of Anti-Protein Sera.

MERRILL W. CHASE. (Introduced by K. Landsteiner.)

From the Rockefeller Institute for Medical Research, New York City.

As reported recently, the serum of guinea pigs rendered hypersensitive to certain chemicals contains antibodies which can transfer to the skin of normal guinea pigs reactivity towards the chemical and towards protein conjugates of the incitant.[1] Sera prepared against proteins have been tested in analogous manner with the idea that the principle might be of wider applicability. This is actually the case. Positive results were first seen with serum from guinea pigs injected repeatedly in the skin with horse serum (0.1 cc of 1:5 dilution in saline); more active sera were frequently secured by a method devised by Dr. Jules Freund as having a substantial adjuvant effect with several antigenic materials,[2] namely, injection under the skin of an emulsion of horse serum, Aquaphor, and killed tubercle bacilli in paraffin oil, the animals being bled 4 or preferably 6 weeks or longer afterwards. When skin sites were prepared in the manner of a Prausnitz-Küstner transfer with 0.15 to 0.05 cc of selected sera, and after an interval of 10 hours to several days the corresponding antigen was injected into the sensitized sites, or subcutaneously at a distance, the skin over and contiguous to these sites gave specific reactions of the early type with distinct erythema and oedema (Table I), as described for the drug hypersensitivity sera.[1]

With a given serum, the extent of the reaction varies markedly with the particular recipient. Thus the reaction of recipient A although typical in its course was in its extent characteristic of the best acceptors; on the average, diameters of 2.5 to 3 cm were attained. The erythema mostly recedes in less than an hour, while the oedema continues to increase for a time before slowly regressing. There may occur some resurgence in color between the 4th and 6th hour. On the following day the skin usually appears normal, but faint, rarely moderate, reactions have been noted at times, chiefly with anti-protein sera.

While the immune sera can often be diluted up to 8- or 12-fold, relatively large amounts of antigen seem preferable for subcutaneous injection. With the latter method of testing, the onset of the skin reactions varied between 10 and 30 minutes, apparently principally dependent upon the attainment of a sufficient concentration of the antigen in the reactive skin area.

Like effects were also seen with selected guinea pig anti-ragweed (*Ambrosia elatior*) sera, obtained after injection of pollen incorporated in an emulsion of the Freund type[2] or by repeated intracutaneous injection of extracts precipitated with alum (Caulfeild[3]), the recipient guinea pigs being tested with the usual "5%" extracts in Coca's fluid (phenol omitted) of defatted pollen, which were injected locally or subcutaneously (Table II). The intensity of the reactions, which started within 4 to 15 minutes, was rather more variable in individual recipients than with other types of sera, possibly indicative of low antibody content. Under the same experimental conditions a few attempted transfers of human reaginic (low ragweed) sera to guinea pig skin have been unsuccessful.

Positive skin transfer effects, elicited by tuberculin, were encountered with sera from guinea pigs which were injected with killed tubercle bacilli in paraffin oil and at the same time with a conjugated antigen. This reaction is being studied and will be described later.

In further experiments it was found that ordinary precipitin sera produced in rabbits

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[1] Landsteiner, K., and Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 688.

[2] Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548; Freund, J., paper read April 2, 1942, before the American Association of Immunologists in Boston, Mass.

[3] Caulfeild, A. H. W., *J. Allergy*, 1936, **7**, 451.

TABLE I.*

Local Sensitization of Normal Skin by Sera from Guinea Pigs Immunized with Horse Serum. The recipients (albino guinea pigs) were prepared with 0.15 cc amounts of immune and normal sera injected intracutaneously on separate halves of the back.

Recipient A 2 days later, 0.5 cc horse serum inj. subcut. on belly Reactions on injected sites			Recipient B 1 day later, intracut. inj. of 0.1 cc horse serum† into same sites Reactions on injected sites		
Time	Anti-horse serum No. 1	Normal G.P. serum	Time	Anti-horse serum No. 2	Normal G.P. serum
13′	A few very faint pink spots about locus of antibody injection	nil	0′	10x10 wheal, almost colorless	10x10 al.cls. wheal
17′	Many pink spots over area 50x35	,,	2′	Wheal has gray-blue hue	
25′	Confluent patches, giving pink, elevated area 50x35, with marginal pseudopodia; tiny pink dots beyond margin	,,	8′	Pink pseudopodia extending outward from wheal	
31′	60x50 pink, markedly elevated	,,	10′	21x21 pink, elevated centrally; uneven, spreading margin	12x10 faintly pink, elevated
38′	Color fading, oedema unchanged	,,	18′	24x24 pink, elevated; center markedly elevated	No change
190′	Colorless; oedema receding	,,	30′	28x28 color receding, still swollen	,, ,,
overnight	Negative	,,	overnight	23x21 faintly brownish pink color	13 faint brownish pink color

* In this and the following tables, the reaction areas are described as to size (cross diameters given in mm), erythema, and oedema.

† Smaller doses are adequate in testing.

TABLE II.

Local Sensitization of Guinea Pig Skin by Guinea Pig Anti-ragweed Serum.
Recipient C.
1 day after the serum injections shown, intracutaneous injections of 0.025 cc "5%" extract of low ragweed pollen into same sites.

	Preparation of sites	
Time	Guinea pig anti-ragweed serum (0.15 cc)	Control serum: Serum from G.P. sensitized to citraconic anhydride (0.15 cc)
8′	18x18 very faintly pink	nil
12′	20x18 pink	,,
35′	23x21, pink, markedly elevated	,,
ca. 45′	Color fading, oedema persistent	,,
overnight	Practically normal	,,

can be employed for local sensitization of guinea pig skin, and with these some of the largest and most pronounced reactions have been observed (transient, marked blue (cyanotic) discoloration over the central part of the area, urticaria-like welts); however, powerful antisera have had to be diluted for the preparatory skin injection in order that typical reactions could be elicited subsequently in the skin. The reason for this may be found to lie in a zone phenomenon or interference by a systemic reaction attributable to that portion of the antibody which escapes from the skin site into the circulation. The rabbit immune sera tested, all with positive skin transfer effects, have been: horse serum precipitin (Table III), anti-hen ovalbumin, anti-"picryl ovalbumin" (tested vs. picrylated casein), anti-pneumococcus III (vs. pneumococcus III polysaccharide*). A horse anti-

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* The author is indebted to Drs. O. T. Avery and W. F. Goebel for making the polysaccharide available.

TABLE III.
Local Sensitization of Guinea Pig Skin by Rabbit and Guinea Pig Antisera (Prepared Against Horse Serum).
Recipient D.
On the day following the intracutaneous injections indicated, 0.5 cc horse serum was injected subcutaneously on the belly.

	Preparation of sites		
Time	Rabbit anti-horse serum .15 cc 1:8 dil. in saline	Guinea pig anti-horse serum No. 3 .15 cc undil.	Normal G.P. serum .15 cc undiluted
37′	25x25 pink, markedly swollen	Trace	neg.
44′	26x26 pink, markedly swollen	19x16 pink, markedly elevated	”
66′	color fading, markedly elevated	24x23, color slightly faded, well elevated	”
overnight	Practically normal	Practically normal	”

pneumococcus III serum did not show the effect.

It may be mentioned that no reactions were obtained in the skin of rabbits (on the ear or the back) in sites injected with rabbit or guinea pig immune sera.

It would seem probable that the reactions described are due to anaphylactic antibodies; so far it can be said merely that in guinea pigs which had received guinea pig antisera intraperitoneally lethal shock could be induced not only in the case of horse serum but also of ragweed.[†]

By testing several like antisera simultaneously in one recipient, the antigen being given subcutaneously, it is possible to evaluate their comparative strengths.

[†] Passive anaphylactic transfer with guinea pig anti-ragweed serum was demonstrated following intraperitoneal injection (6 cc) into a normal guinea pig of 330 g weight, typical shock and death ensuing within 4 minutes following intravenous injection of 0.5 cc “5%” extract on the next day. The extract was not at all toxic for a normal control.

14103

Alterations in Response to Changing Body Temperature Following Artificial Fever and Chilling.

WALTER C. RANDALL.* (Introduced by W. A. Hiestand.)

From the Department of Animal Physiology, Purdue University, Lafayette, Indiana.

During investigations upon the temperature control of birds, modifications of both panting and shivering temperature thresholds due to artificial fever and chilling were studied. Both young and adult domestic fowls (*Gallus domesticus*) were first placed in an ice chamber in which the temperature averaged about 10°C, and then in a heating blanket in which the temperature averaged about 55°C. After a suitable recovery period, the order of the exposure was reversed, *i.e.*, the same bird was first warmed and then chilled. During these changes in body temperature, changes in breathing rate were recorded. Temperatures were recorded from sensitive thermometers placed to a constant depth in the cloaca and under the skin. The first muscular tremors preceding continuous shivering were detected by a stethoscope placed upon the muscles of the limbs and breast.

Eighty minutes were required to lower the temperature of a typical bird (Table I) one

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[*] Now with Department of Physiology, Saint Louis University, Saint Louis, Mo.

TABLE I.
Lowered Panting Temperature Threshold Following Chilling.

Time in min.	Cloacal temp. °C	Skin temp. °C	Environmental temp. °C	Breathing rate/min	Remarks
0	42.1	41.1	25.0	28/min.	
10	41.8	40.5	10.0	34	Muscle tremors
30	41.6	39.6	”	44	
60	41.2	39.6	”	36	Severe shivering
100	40.7	38.9	”	38	
135	39.1	37.6	”	36	
		Transferred to Heating Blanket.			
138	39.0	38.8	55.0	30	
150	39.3	40.2	”	28	
180	40.9	41.5	”	36	
195	41.7	42.3	”	60	Pre-panting rate
206	42.1	42.8	”	120	Panting rate

Condensed protocol showing a lowered panting temperature threshold in the domestic fowl after preliminary chilling.

TABLE II.
Depression of Shivering Following Warming.

Time in min.	Cloacal temp. °C	Skin temp. °C	Environmental temp. °C	Breathing rate/min	Remarks
0	42.0	41.4	25.0	36/min	
10	42.2	42.6	55.0	72	
20	42.7	43.2	”	160	Panting rate
40	42.9	43.3	”	152	
70	43.1	43.6	”	145	
		Transferred to Ice Chamber.			
72	43.0	42.6	10.0	96	Reflex inhibition
80	41.6	41.2	”	48	
90	41.0	40.2	”	28	
95	40.8	39.9	”	40	Muscle tremors
112	40.0	39.2	”	36	Severe shivering
130	39.6	38.6	”	36	
160	39.0	37.6	”	36	

Condensed protocol showing depression of shivering responses following preliminary warming in the domestic fowl.

degree. A total fall of 3 degrees was produced in 135 minutes. Shivering in the preliminary form of periodic muscular tremors appeared promptly and became more severe as chilling continued. The breathing rate increased somewhat upon exposure to the cold packs, and remained rapid throughout the period of chilling. After the body temperature had been maintained at a subnormal level for a short period, it was quickly raised to the normal level. As the body temperature approached normal however, breathing began to increase much as it does in birds approaching the lower threshold limit of panting. In fact, the rate became polypneic such as is characteristic of normal birds while panting by the time the original body temperature was reached. In other words, a lower panting temperature threshold was established. In many birds the panting rate was 6 to 8 times the normal rate when the body temperature was returned to the pre-chilling level.

To ascertain whether the threshold of shivering could be reciprocally affected by preliminary heating, the same birds were first placed in the heating blanket and later transferred to the ice chamber. Panting occurred after the deep cloacal temperature rose to the panting threshold, increased to a maximum rate, and then decreased to a slightly slower rate. After a period of 50 minutes of hyperthermia the birds were transferred to the ice chamber. The breathing rate decreased suddenly from 145 to 96 breaths per minute, even before the deep cloacal temperature had started to fall indicating definitely reflex inhibition of panting. The body temperature quickly fell to the normal level, and continued

TABLE III.
Panting and Shivering Responses Before and After Artificial Fever and Chilling.

Bird No.	Starting temp. °C	(High) (Low) temp. °C	Length of exposure	Normal panting threshold	After chilling	Normal shivering threshold	After heating
Domestic Fowl							
50	41.4	44.7	45 min.	42.3	41.2		
		38.0	90			39.3	37.1
46	42.0	43.8	73	42.4	41.7		
		40.6	125			42.0	41.5
47	42.4	44.8	70	43.2	41.8		
		40.2	150			42.3	40.6
49	41.4	45.0	55	42.8	40.3		
		38.8	70			40.7	37.8
45	42.0	43.1	70	42.7	42.0		
		39.0	138			41.7	40.8
Pigeons							
A	41.6	45.0	30	42.4	40.6		
		37.5	50			40.6	38.5
D	42.2	44.7	65	43.1	40.8		
		22.0	135			41.5	40.9

to fall an additional 3 degrees in 85 minutes, this fall corresponding to a similar fall in 135 minutes in the same bird without preliminary heating. Shivering was delayed and did not become severe until a threshold considerably below that of the normal bird was reached. (Table II.)

Discussion. It is thus indicated that as a result of previous chilling, the "panting thermostat" is reset at a new and lower level so that the panting rate of breathing is initiated at temperatures below the normal level (Fig. 1). After preliminary heating, shivering is delayed and does not reach the same intensity as that of the normal bird. Heat therefore actually depresses the sensitivity (raises the threshold) of the shivering mechanism, resulting in a more severe and rapid fall of body temperature. Table III summarizes these alterations as well as the limits of variation in several birds chosen as typical of all experiments.

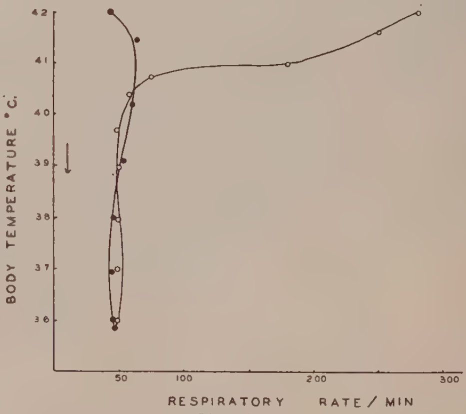

FIG. 1.
Alteration of the panting mechanism following severe hyperthermia, as demonstrated by a lowered panting temperature threshold. Note that panting begins on the ascending limb of the curve between 40° and 41°C but was absent on the descending limb at 42°C. Solid circles indicate decreasing body temperature; open circles indicate rising temperature.

Summary. Alteration of the temperature regulating mechanism of the fowl has been demonstrated by a lowered panting temperature threshold following a maintained reduction of body temperature (3 degrees for 2 hours). Maintenance of body temperature in a condition of hyperthermia for 1 to 2 hours previous to chilling does not result in a reciprocal change in the shivering threshold, rather, heat acts as a depressant and decreases the sensitivity of the shivering mechanism under these conditions. Reflex inhibition of panting with application of cold to the skin is demonstrated.

14104

Assay of Tryptases by Lysis of Fibrinogen.

John H. Ferguson.

From the Department of Pharmacology, University of Michigan, Ann Arbor.

A recent method[1,2] for assay of tryptases by lysis of *fibrin* clot is sufficiently sensitive to give excellent results with normal dog, cat and human plasma tryptases, which, in a limited series of observations to date, fall within the range of 20-200 "units" (*v. infra*), in which the test is accurate to within 5-10%. The principle of the method is the comparison of the "lytic times" of activated tryptase "unknown" with a standard reference series of known trypsin concentrations. A convenient practical *standard* of 100 "units" is defined as the lytic activity of 1 mg per cc of a reliable commercial trypsin* (prepared by dilution of a "stock" 2% solution preserved in glycerol-borate buffer, according to Burdon[3]). Since some species (*e.g.* rabbit[4]) and certain human bloods yield plasma tryptase values under 5 "units" by the above test, a new method has been devised to operate with a sensitivity of ten times that of the original test. It has the same (5-10%) experimental error in the new range of 10-1 "units." Like a number of older attempts (*e.g.*[5]), the new method involves lysis of *fibrinogen*. This is timed by testing the residual clotting activity with a potent thrombin. As in the fibrinolytic method, the assay depends upon comparison of the "unknown" with a series of standard enzyme dilutions.

Reagents. 1. *Simplified borate buffer solution.* 2.5% H_3BO_3 (45 parts), 0.5% NaCl (45 parts), 4% $Na_3B_4O_7,10H_2O$ (10 parts): pH $= 7.7 \pm 0.1$ (glass electrode). This solution is used as solvent and diluent for fibrinogen and thrombin.

2. *Fibrinogen.* A lyophilized preparation[6] is convenient and an especially pure preparation (about 50% coagulable protein) of human fibrinogen, prepared by Dr. R. M. Ferry and the staff of the Harvard Plasma Fractionation Laboratory and kindly supplied by Dr. E. J. Cohn[7] for this work, is excellent. Essential qualities are (a) stability, denoting freedom from contaminating protease, (b) absence of natural trypsin inhibitors. It is evident that these requirements are fulfilled in the data of Table I. Series B of this table illustrates the clotting-times of this fibrinogen, serially diluted with buffer solution. These values could be used to convert the clotting-times of Series A into terms of residual substrate (a topic not germane to the present discussion). Their routine value, however, is to test the fibrinogen in order to be sure that the initial strength, while giving good solid clots, is such that significant prolongation of clotting-time accompanies reduction of fibrinogen concentration to, say, 25-10% of the original, an obvious requirement for a sensitive lytic test. 1:250 or 1:200 solution of purified human fibrinogen meets these specifications.

3. *Thrombin.* This must be stable in the test solution. An excellent preparation consists of a 1:25 dilution (with borate buffer) of Parfentjev's[8] "hemostatic (clotting) globulin" (Lederle). As now marketed, this material contains preservatives which appear to inhibit proteases, making it unsuitable for our fibrinolytic (original) method. Since the new technic involves addition of the thrombin only *after* the enzyme action, this inhibitor, if anything, is an advantage, in that it tends to arrest the enzyme action at the start of the

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[1] Ferguson, J. H., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 73.

[2] Ferguson, J. H., *Federation Proc.*, 1943, in press.

* Fairchild Bros. and Foster.

[3] Burdon, K. L., *Science*, 1941, **93**, 91.

[4] Ferguson, J. H., *J. Lab. and Clin. Med.*, 1943, in press.

[5] Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 255.

[6] Ferguson, J. H., and Erickson, B. N., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 425.

[7] Cohn, E. J., *Chem. Rev.*, 1941, **28**, 394.

[8] Parfentjev, I. A., *Am. J. Med. Sci.*, 1941, **202**, 578.

TABLE I.
Trypsin Assay by Lysis of Fibrinogen.
Clotting-times (sec) for 0.5 cc thrombin + 1 cc samples of digesting mixtures, *viz.*, 1:250 fibrinogen (10 cc) + enzyme (1 cc).

A. Digestion series:

Enzyme conc.*	Minutes of incubation time (pH = 7.7, temp. = 25°C) during										
	5	10	15	20	25	30	60	90	120	150	180 min.
10	43	360	∞								
9	20	38	85	540	∞						
8	19	32	64	240	∞						
7	18	27	40	80	135	∞					
6	17	23	31	48	88	180	∞				
5	15	18	—	22	—	27	48	150	420	∞	
4	15	16	—	19	—	23	35	55	105	270	600
3	15	15	—	17	—	20	29	45	72	155	360
2	15	15	—	16	—	17	20	23	25	30	35
1	15	15	—	15	—	15	17	18	18	19	22
0	15	—	—	—	—	—	15	—	15	—	15

B. Fibrinogen dilution series:

Fibr. dilu.	1:1	1:2	1:4	1:8	1:16	1:32	1:64
Mg per cc	4	2	1	0.5	0.25	0.125	0.063
Clot-time	15″	16″	18″	23″	45″	140″	trace

*1 mg per cc of "standard" trypsin (see text) = 100 *units*.

clotting test.

4. *Standard Trypsins.* A series of dilutions varying by 1 unit, between 10 and 1 unit, is made by diluting "stock" trypsin (*v. supra:* 2% solution = 2000 "units" per cc) with physiological saline.

5. *Unknowns.* Some materials, *e.g.*, prostatic fluid, appear to contain the protease in active form, since no significant change in activity is produced by treatment with chloroform. Plasmas, sera, etc., normally contain excess of *trypsin-inhibitor*, which has long been known to be removed (hence "activating" the enzyme) by shaking with $CHCl_3$ (1/5 vol.), removing the latter by centrifuging after 24-48 hours (*cf.*[9]).

Tests. Digestion of fibrinogen: 10 cc fibrinogen (*v.* reagents) is incubated with 1 cc enzyme; 1. standard trypsins, 2. unknown(s)—suitably activated and diluted (if indicated, by preliminary test). Arbitrarily selected "standard" conditions include: (a) pH = 7.7 ± 0.1 (*v.* simplified buffer) to control pH and ionic strength, (b) temp. = 25° ± 2°C. Usually, room temperature is sufficiently constant but a thermostat water-bath may be a desirable refinement.

Assay series. 1 cc samples of fibrinogen-enzyme mixture are added to 0.5 cc of thrombin (*v.* reagents) in Wassermann tubes and the presence or absence of clotting noted. For a simplified test, the following designations suffice: + : solid clot (tube invertible), ± : incomplete clot, ∞ : no clot or mere trace of flocculation. Meticulous accuracy is obtained by actual timing of the *onset* of fibrin formation, as in Table I. Times of sampling (incubation periods) will be determined by strengths of enzyme under consideration. Comparison of the "lytic time" of unknown with standard enzymes enables assay of the former in terms of the "units" expressed by the latter.

Data. Table I illustrates the sensitivity and selectivity of the trypsin standards, 1 "unit" representing the equivalent of 1:100,000 trypsin (0.01 mg per cc of enzyme solution) or somewhat less than 1:1,000,000 trypsin in the final digestion mixture.

Table II illustrates the practical application of the test to the assay of the tryptase content of a sample of dog prostatic fluid, kindly supplied by Dr. C. Huggins (Chicago).[10] The value of 5 units is seen to be obtained both

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[9] Tagnon, H. J., *J. Lab. and Clin. Med.*, 1942, **27**, 1119.

[10] Huggins, C., and Neal, W., *J. Exp. Med.*, 1942, **76**, 527.

TABLE II.
Routine Assay of Tryptase in Dog Prostatic Fluid.
A. Untreated; B. $CHCl_3$-treated.

Enzyme conc.	Incubation period 5′	15′	20′	30′	60′	75′	90′
Sample A	+	+	+	+	+	±	∞
Sample B	+	+	+	+	+	±	∞
10 units	+	∞					
5 ”	+	+	+	+	+	±	∞
2.5 ”	+	+	+	+	+	+	+

with untreated and $CHCl_3$-treated fluid.

Discussion. Numerous applications of the method to the assay of tryptic enzymes in plasma, serum, cell- and other materials are envisaged. The effects of inhibitors and other conditions modifying the action of known tryptases also lend themselves to quantitative investigation by simple modification of the cited technic. The new method (which may be styled Method B) offers the following advantages over the original[1] assay (Method A): 1. tenfold increase in sensitivity; 2. independence of enzyme-inhibitors in thrombin; 3. much less time-consuming—a matter of minutes or hours (actual test), instead of days, even with very weak enzymes; 4. sharper "end-point," since the enzyme acts in water-clear homogeneous colloidal solution, instead of on the grossly biphasic fibrin gel; 5. no special apparatus (*e.g.*, turbidimeter) required. Within their respective ranges and limitations, both methods are useful practical means of estimating natural trypsin-like proteolytic enzymes. Such enzymes play an important role in blood-coagulation[11] and other functions, to the elucidation of which it is believed that these technics may offer a material contribution. The methods are sufficiently simple to open up new fields as a clinical laboratory test.

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[11] Ferguson, J. H., *Science*, 1943, in press.

14105

Toxemias of Pregnancy and the Inulin-Diodrast Clearance Tests.

Donald H. Kariher and Roger H. George. (Introduced by E. F. Adolph.)

From the Department of Obstetrics and Gynecology, Strong Memorial and Rochester Municipal Hospitals, University of Rochester School of Medicine and Dentistry.

The role of the kidneys in the production of toxemia of pregnancy has been studied for many years, and as yet we have only a paucity of information. As one new kidney function test after another has been announced, investigators have applied them to the toxemias of pregnancy and have found them inadequate to detect alterations in the status of the kidneys.

It would seem so even with the newest of function studies, the inulin and diodrast clearance tests. Chesley *et al.*[1] conclude that the renal blood flow is essentially normal in eclampsia, pre-eclampsia, and recurrent toxemia of pregnancy, as measured by the diodrast clearance. Corcoran and Page[2] feel that if the filtration fraction is

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[1] Chesley, L. C., Connell, E. J., Chesley, E. R., Katz, J. D., and Glissen, C. S., *J. Clin. Invest.*, 1940, **19**, 219.

[2] Corcoran, A. C., and Page, I. H., *Am. J. Med. Sc.*, 1941, **201** n. s., 385.

TABLE I.

Averages of Tests Done Ante-partum and up to 12 Days Post-partum.

No. of tests	Type of case	Plasma clearance of Inulin, cc/min	Plasma clearance of Diodrast, cc/min	Filtration fraction, %	Total renal blood flow, cc/min
8	Normal	150	754	20.9	1208
7	Mild pre-eclampsia	109	579	19.0	938
7	Severe pre-eclampsia	115	531	21.4	879
3	Eclampsia	83	426	18.9	641
8	Hypertensive disease	145	613	23.6	990
	Averages of Tests Done More Than 12 Days Post-partum.				
2	Normal	141	653	21.7	1097
7	Mild pre-eclampsia	121	553	21.9	901
9	Severe pre-eclampsia	138	612	21.9	1064
4	Eclampsia	112	539	20.0	938
6	Hypertensive disease	118	521	24.8	937

increased, the patient is not truly toxemic but rather pre-existingly or latently hypertensive, and that only those cases showing normal clearance can be classified as true toxemias. It is generally agreed[3,4,5] that hypertensives show a decrease in total renal blood flow, with an elevated filtration fraction. However, hypertension with pregnancy is not a toxemia, it represents merely the co-existence of an abnormal with a normal condition in the same patient.

Welsh *et al.*[6] studied 21 normal women, some of whom were tested both before and after delivery, by means of inulin, phenol red, and diodrast clearance tests. They conclude that kidney function is not altered in pregnancy nor in the puerperium. It is to be noted however that they record a slight decrease in the plasma clearances of both inulin and diodrast in the post-partum phase as compared to the ante-partum. These same authors[7] using the same methods as above, and studying toxemic patients, believe that the inulin clearance is decreased during toxemia, but not sufficiently for the results to be out of the range of normal. The diodrast clearance is normal or above normal in the ante-partum tests with a slight fall post-partum. The filtration fraction consequently is reduced ante-partum, and rises to and above normal post-partum. They believe that if clearance studies are done ante-partum and post-partum some aid as to the ultimate prognosis of the disease may be obtained.

We wish to report our findings on pregnant and post-partum women who have had one of the toxemias of pregnancy, and whom we studied with the inulin and diodrast clearance tests.

Methods. The patients used in this study were women who were under treatment for some form of toxemia of pregnancy, or who were admitted to the hospital specifically for this study. The procedures of the test are described by others.[8,9] The clearance figures in all the tables are corrected to a surface area of 1.73 sq m using the DuBois Body Surface Chart.

Patients were classified as to their toxemia according to the prerequisites laid down in 1939 by the American Committee on Maternal Health.[10]

Results and Discussion. Our findings are summarized in the tables. A total of 52 patients were studied. Table I presents the average results found in all the studies, and

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[3] Foa, P. P., Woods, W. W., Peet, M. M., and Foa, N. L., *Arch. Int. Med.*, 1942, **69**, 822.

[4] Chesley, L. C., and Chesley, E. R., *J. Clin. Invest.*, 1940, **19**, 475.

[5] Friedman, M., Selzer, A., and Rosenblum, H., *J. A. M. A.*, 1941, **117**, 92.

[6] Welsh, C. A., Wellen, I., and Taylor, H. C., Jr., *J. Clin. Invest.*, 1942, **21**, 57.

[7] Wellen, I., Welsh, C. A., and Taylor, H. C., Jr., *J. Clin. Invest.*, 1942, **21**, 63.

[8] Smith, H. W., Goldring, W., and Chasis, H., *J. Clin. Invest.*, 1938, **17**, 263.

[9] White, H. L., and Rolfe, D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 1.

[10] Stander, H. F., *Am. J. Surg.*, 1940, **48**, 112.

TABLE II.
Eclampsia.

Case No.	Test ante-partum mo.	Test post-partum		Max. B.P.	Max. urinary alb., g 24 hr	Duration of alb., days	Casts	Hematocrit, %	Plasma clearance of		Filtr. fract., %	Total renal bl. flow, cc/min
		days	mo.						Inulin, cc/min	Diodrast, cc/min		
1 E		10		188/144	7	?	4-5	31	121	554	21.8	748
2 E		14		154/110	8	?	5	38	86	445	19.2	719
3 E			18	?	?	?	?	41.5	181	903	20	1544
4 E	1½			170/116	12	14	0	39.0	44	279	15.7	457
			4	120/88	tr	120	0	38.0	35	220	16.1	355
5 E			17	178/?	?	?	0	43.0	116	597	19.5	1048
6 E		23		204/130	9	?	Occas.	41.5	96	472	20.3	808

further compares the average values obtained on patients tested during pregnancy and up to 12 days post-partum with those obtained after the puerperal period. In the normal patients there is a slight decrease in both inulin and diodrast clearance in those patients studied post-partum as compared to the results in the patients studied ante-partum. This finding is essentially the same as that recorded by Welsh *et al.*[6] We note a slight decrease in the glomerular filtration rate and total renal blood flow in both the mild and severe pre-eclamptics, but the values still are within normal limits. It is only in the eclamptic patients studied during or immediately after their eclampsia that any appreciable change is seen in the kidney function. In all these cases the filtration fraction is within normal limits. In the hypertensive of course we obtain the usual fall in glomerular filtration rate and total renal blood flow with a rise in filtration fraction.

Although the series of cases of eclampsia is small, the results recorded in Table II are of interest. The interval elapsed between the time of the eclampsia and the time of the test seems to be of some importance. In cases No. 1E, 2E, 4E and 6E where the interval is short the clearance values are definitely subnormal, whereas in cases No. 3E and 5E, where the interval is longer, normal results are obtained. This would lead one to speculate that there is some malfunction of the kidneys during and for a short time after the toxemia, but that the malfunction is not the result of permanent kidney damage, since the function later returns to normal. Just what the nature of the functional abnormality of the kidneys is, is difficult to postulate since the filtration fraction remains normal throughout.

Case No. 4E is of particular interest, and is possibly an example of pregnancy complicated by nephritis with convulsions, rather than one of pure eclampsia. There is no clinical history of previous renal disease, and the pregnancy was uncomplicated until about 2 months from term at which time the blood pressure began to rise and albumin was first detected in the urine. Function studies done shortly after the onset of clinical symptoms showed a definite decrease in renal function, which is of course highly suggestive of previous renal damage. Very similar results were recorded some 4 months after delivery. Some 12 months after delivery the patient still had a blood pressure of 136/90 and a trace of albumin in the urine. It is to be noted in this case that the filtration fraction is quite low. This may also be indicative of previous kidney disease.

Summary. Results of inulin and diodrast clearance tests performed on 52 pregnant or post-partum females are presented. Eclampsia is the only toxemia which sometimes shows a decreased kidney function as evidenced by a diminution in the clearance of inulin and diodrast. The filtration fraction is normal in all toxemias of pregnancy.

14106

Prevention of Crystalluria During Sulfadiazine Therapy. Experimental and Clinical Studies.

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From The New York Hospital and Department of Medicine, Cornell University Medical College, New York City.

While with the increasing use of sulfadiazine the incidence of all toxic reactions has remained low, the seriousness of "renal irritation" has assumed greater importance ([1–7] and others). Most authorities agree that in the majority of, if not in all, instances this reaction is produced by the precipitation of sulfonamide compound in the kidneys and ureters. For this reason, it seemed important to make further studies of the solubilities of sulfadiazine and of acetyl sulfadiazine at varying pH levels, and to observe the effect of urinary pH on the incidence of crystalluria in patients receiving sulfadiazine.

Methods. An amount of sulfadiazine or acetyl sulfadiazine* in large excess of that to be dissolved was added to M/15 phosphate buffer solutions ranging in pH from 5.2 to 8.0. The mixtures were shaken for 18 hours in a water bath at 37°C, and filtered at the same temperature. The pH of the filtrate was measured immediately with a Beckman pH meter and appropriate temperature corrections were made. Similar solubility studies were made in M/10 citrate plus NaOH buffers, and in 2 concentrated, acid urines from normal subjects. These urines were divided in aliquots and adjusted in pH from 5.2 to 7.6 with small amounts of alkali. Free and acetylated sulfadiazine were measured according to Bratton and Marshall.[8]

First morning specimens of urine were collected on various services of the hospital from patients receiving sulfadiazine orally. The urines were kept at room temperature and examined within a few hours after they were voided. The urinary pH value was estimated with nitrazine paper, and the sediment after centrifugation was examined microscopically for the presence of crystals due to the drug.

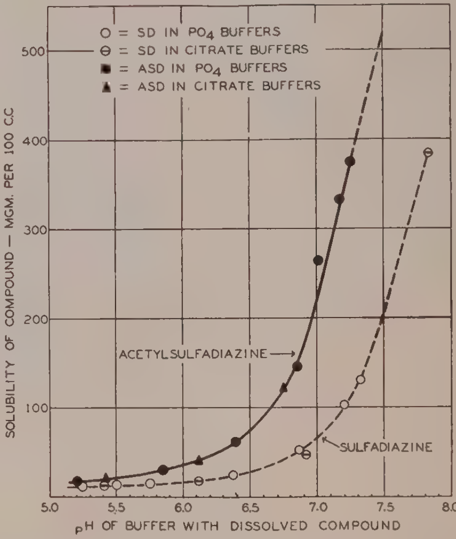

Fig. 1.

The curves of solubility of sulfadiazine and of acetyl sulfadiazine in phosphate and in citrate buffer solutions of varying pH's.

Results and Discussion. The solubilities of

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[1] Finland, Maxwell, Strauss, E., and Peterson, O. L., *J. A. M. A.*, 1941, **116**, 2641.

[2] Thompson, G. J., Herrell, W. E., and Brown, A. E., *Proc. Mayo Clin.*, 1941, **16**, 609.

[3] Bradford, H. A., and Shaffer, J. H., *J. A. M. A.*, 1942, **119**, 316.

[4] Schulte, J. W., Shidler, F. P., and Niebauer, J. J., *J. A. M. A.*, 1942, **119**, 411.

[5] Hellwig, C. A., and Reed, H. L., *J. A. M. A.*, 1942, **119**, 561.

[6] Keitzer, W. A., and Campbell, J. A., *J. A. M. A.*, 1942, **119**, 701.

[7] Plummer, Norman, and Wheeler, C., to be published.

* The sulfonamides were supplied by Lederle Laboratories, Inc.

[8] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

sulfadiazine and of acetyl sulfadiazine increase markedly with rise in pH from 5.0 to 8.0. At a given pH the solubilities of these compounds are essentially the same in phosphate and citrate buffers (Fig. 1) and, under the express conditions of these experiments, in normal urine. Rose *et al.*[9] by varying the pH with addition of 0.1 N sodium hydroxide to the compounds dissolved in water have demonstrated also that the solubilities of sulfadiazine and acetyl sulfadiazine in water increase markedly between pH 5.5 and 7.5. Their experimental procedure yielded results somewhat different from ours, however. Urinary crystals and renal stones from patients receiving sulfadiazine orally appear from analysis to be chiefly acetyl sulfadiazine[3,6,10] and the solubility at varying pH's in phosphate buffers of the acetyl portion[†] of these urinary crystals is similar to that of pure acetyl sulfadiazine.[10] The large increase in solubility of acetyl sulfadiazine in buffers with increasing pH (Fig. 1) is to be contrasted with no change in solubility for acetyl sulfapyridine[9] and a comparatively small change for acetyl sulfathiazole[11] within the same pH range.

TABLE I.
Effect of Urinary pH on Incidence of Crystalluria from Sulfadiazine in 73 Patients Receiving 4-6 g of Sulfadiazine Orally per Day, with or without Varying Doses of Sodium Bicarbonate.

	Acid urines	Neutral or alkaline urines
No. of specimens	172	147*
No. of specimens with crystals	47	2
% of specimens with crystals	27	1.4

* 132 of these 147 urine specimens were from patients receiving alkali therapy.

Clinical studies which were suggested by the above solubility findings revealed the incidence of crystalluria following sulfadiazine therapy to be markedly higher in acid than in alkaline urines (Table I): 27% of 172 acid urines and only 1.4% of 147 neutral or alkaline urines. Factors other than pH which might affect the incidence of crystalluria—namely, the daily urinary volume, the specific gravity of the urine specimen examined, and the daily dose of sulfadiazine—were essentially the same in both groups. Nine patients who had had crystalluria in acid specimens no longer had crystals after sufficient sodium bicarbonate was administered to render the urine neutral or alkaline. In 2 patients showing crystals in acid urines, crystals were again found on the first morning the urine was rendered alkaline by alkali therapy, but were not present subsequently with a maintenance of alkaline urines. These 2 instances are the only ones in which we observed crystals in alkaline urine (Table I). It may be that these crystals were formed when the urine was acid and were being washed out of the urinary tract subsequently. Schwartz *et al.*[12] found an incidence of 25% crystalluria in 108 acid urines and 12% in 33 neutral or alkaline urines from patients receiving 4 g of sulfadiazine daily.

The dosage of sodium bicarbonate necessary to maintain the urine neutral or alkaline during sulfadiazine therapy is, of course, variable. With no alkali therapy, approximately 90% of the urine specimens are acid[12] (our data). Our experience to date, and the data of Schwartz *et al.*[12] indicate that less than 12 g daily of bicarbonate is usually insufficient. In a carefully controlled study of a group of 15 medical patients receiving an initial dose of 6 g of sodium bicarbonate followed by 19.5 g of the alkali daily, divided into 6 doses of 3.25 g (50 grains), all of the 44 urine specimens examined were pH 7.0 or over. In a similar study of 39 urines from 10 medical patients receiving an initial dose of 4 g of sodium bicarbonate followed by 13.7 g daily, divided into 6 doses of 2.3 g (35 grains), the urines were usually pH 7.0 or over but occasionally (6 specimens) were pH 6.3 to 6.8. No crystalluria was observed in either of these 2 groups. The alkali was well tolerated; none of the patients had gastrointestinal symptoms and there were no clinical evidences of alkalosis. In some instances, such as in renal or cardiac insufficiency, sodium bicarbonate may be contraindicated. It must be emphasized that the purpose of this alkali therapy is to maintain the urine neutral or slightly alkaline, and not to produce alkalosis.

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[9] Rose, F. L., Martin, A. R., and Bevan, H. G. L., *J. Pharm. and Exp. Therap.*, 1943, **77**, 127.

[10] Unpublished data from this laboratory.

[†] The crystals studied yielded on analysis 93% acetyl sulfadiazine and 7% sulfadiazine.

[11] Climenko, D. R., Barlow, O. W., and Wright, A. W., *Arch. Path.*, 1941, **32**, 889.

[12] Schwartz, L., Flippin, H. F., Reinhold, J. G., and Domm, A. H., *J. A. M. A.*, 1941, **117**, 514.

Conclusions. 1. The solubilities of sulfadiazine and particularly of acetyl sulfadiazine, in buffers and in normal urine, increase markedly with increasing pH within the physiological pH range of urine. 2. Examination of urine specimens from patients receiving sulfadiazine has demonstrated that crystalluria due to sulfadiazine can be prevented by maintaining the urine neutral or alkaline and the volume within limits generally considered optimum for patients with infection. 3. This study affords evidence that renal reactions due to the precipitation of sulfadiazine compounds can be prevented by appropriate alkali and fluid therapy. Our clinical findings to date accord with this assumption.

14107

Nutrition of the Golden Hamster.*

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Since several reports have indicated that the Golden or Syrian Hamster (*Cricetus auratus*) may be useful in studies involving human virus diseases,[1,2] we were interested in determining more specifically the nutritional requirements of this animal. It was immediately obvious that the hamster grew very poorly and failed to survive on synthetic diets which allow good growth and reproduction in the rat. The addition of liver extract did not improve the rate of growth appreciably and many animals failed on diets containing several natural products. The animals showed somewhat better growth when the synthetic diet was supplemented with whole liver substance or with preparations from grass.

We wish to report in this paper that one of the limiting factors in purified diets for the hamster is biotin and that when biotin is supplied in adequate amounts together with inositol and para-aminobenzoic acid excellent growth results. Under these conditions neither nicotinic acid nor vitamin C needs to be supplied in the diet.

Experimental. All the animals used were raised in our laboratory from a few stock animals originally obtained from Dr. A. F. Rasmussen, Department of Bacteriology. The stock ration was similar to that used for rats with additional greens and whole corn and wheat. The young were started on experiment at weaning when they weighed 25 to 35 g. The basal synthetic ration was similar to that used in our studies with rats and had the following composition: sucrose 72, casein 18, salts IV 5,[3†] corn oil 2, cod liver oil 2, and wheat germ oil 1. The ration was fed *ad libitum* and 1 drop of haliver oil was given to each animal every 2 weeks. All the vitamins were given in great excess in order to eliminate any possibility that the requirement of

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[*] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was aided by a grant from The National Foundation for Infantile Paralysis, Inc.

We are indebted to Merck and Co., Rahway, New Jersey, for crystalline vitamins; and to Wilson Laboratories, Chicago, Illinois, for whole liver substance and 1-20 powder.

[1] Lennette, E. H., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 178.

[2] Wheeler, A. H., and Nungester, W. J., *Science*, 1942, **96**, 92.

[3] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

[†] Salt mixture of Phillips and Hart modified by an addition of 5 g $MnSO_4$ per kilo.

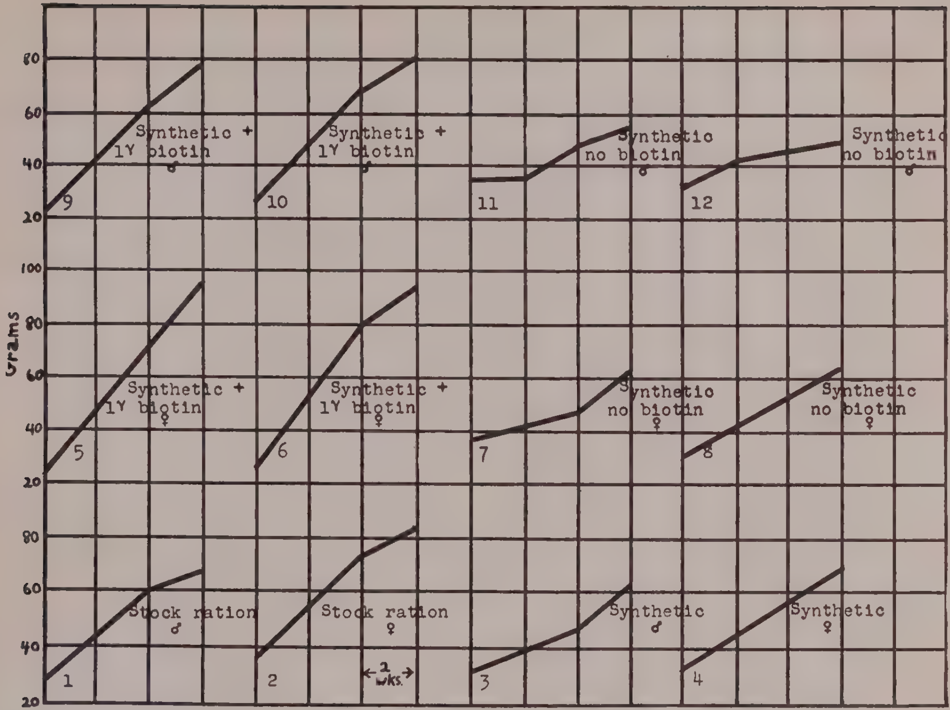

CHART I.

Best pair (3 and 4) on synthetic diet compared with hamsters on stock ration (1 and 2).
Two typical females (5 and 6) receiving biotin compared with 2 typical females without biotin (7 and 8).
Two typical males (9 and 10) receiving biotin compared with 2 typical males without biotin (11 and 12).

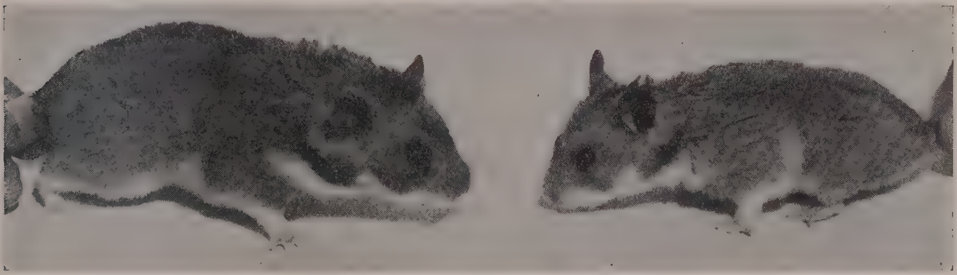

FIG. 1.

Two female litter mates after 7 weeks on experiment. Hamster on left received synthetic diet plus 1 γ biotin daily. Weight 108 g. Hamster on right, synthetic diet with no biotin. Weight 62 g.

the hamster might be higher than for the rat. The daily intake was as follows: thiamine 500 γ, riboflavin 500 γ, calcium pantothenate 500 γ, pyridoxine 500 γ, nicotinic acid 750 γ, sodium para-aminobenzoate 1 mg, inositol 1 mg, and choline 1 mg. The B vitamins were given in 20 cc water in supplement cups daily. In all our early work 1 mg of ascorbic acid was also given each animal daily.

Weanling hamsters placed on the synthetic diet containing the 8 B vitamins and vitamin C grew poorly and many of them died between the 3rd and 5th week after starting on experiment. In one typical series involving 17

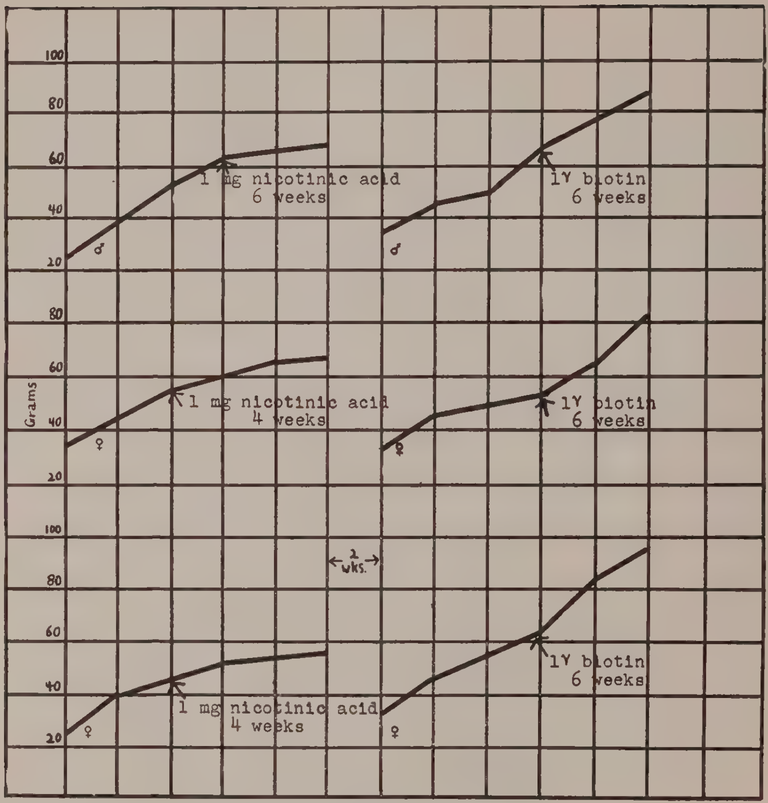

CHART II.

Comparative effects of nicotinic acid and biotin given to hamsters receiving synthetic diet plus ½ γ biotin but no nicotinic acid.

animals, 5 were dead at 4 weeks. The surviving males showed an average daily gain of 0.66 g and the females .95 g during the 4-week period. It is interesting to note that the female hamster grows faster and becomes a larger animal than the male.[4] In Chart I we have compared the growth curves for the best pair of hamsters on the synthetic diet with those for a typical pair on the stock ration. In this case the record approached that of the stock animals but most of the animals on our stock ration have also grown poorly. A few animals from small litters grew fairly well on the synthetic diet for 4 weeks but died suddenly during the fifth and sixth weeks. This higher weight and longer survival time were undoubtedly due to a greater storage of certain vitamins in the animals at weaning. Many of the animals developed a characteristic dermatitis at the corners of the mouth. If the animals lived beyond 6 weeks this dermatitis became fairly extensive.

The addition of 3% liver extract (1-20) prolonged the life of the animals but did not give a significant growth response. Whole liver substance at 3% of the ration produced a growth rate as high as 1.2 g per day for the females. Since liver extract is a good source of "folic acid" and other factors required by the chick these factors could not be the limiting ones for the hamster unless their requirements were exceedingly high. However, whole liver is distinctly higher in biotin than liver extract as shown by microbiological assay and since

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[4] Bruce, H. M., and Hindle, E., *Proc. Lond. Zoo. Soc.*, 1934, 361.

TABLE I.
Comparison of Growth Rates on Synthetic Diets and Stock Ration.

	No.	Sex	g per day 4 wk
Basal + 8 vitamins of B group, no biotin	8	♂	0.66
	7	♀	0.95
Basal + 8 vitamins of B group, 1 γ biotin	7	♂	1.12
	13	♀	1.40
Basal + 7 vitamins of B group (no nicotinic acid) + 1 γ biotin	3	♀	1.30
Basal + 7 vitamins of B group (no nicotinic acid), no biotin	5	♂	.80
	2	♀	.92
Stock ration	2	♂	.90
	3	♀	1.00

the animals showed a dermatitis we suspected a possible lack of this vitamin.

As soon as a biotin concentrate‡ was added to the synthetic diet in amounts sufficient to supply 1 γ of biotin per day remarkably good growth was obtained in all cases and no dermatitis was evident. A few typical results are given in Chart I. With added biotin the males showed an average daily gain of over 1 g per day and the females 1.4 g per day during the first 4 weeks on experiment. The animals continued on to maturity and we have carried a few female animals through reproduction with fair success.

When the animals were given the synthetic diet plus 1 γ biotin daily there was apparently no need for nicotinic acid. In a typical experiment 3 females grew very well on a ration deficient in nicotinic acid but complete in all other respects. They gained an average of 1.3 g per day for the first 4-week period and no dermatitis was evident. When both biotin and nicotinic acid were omitted from the daily vitamin supplement the animals weighed less than the animals on a ration containing both these factors. However, controls getting nicotinic acid but no biotin did no better.

Several of the animals were given the synthetic diet with only 0.5 γ biotin and no nicotinic acid. Poor growth resulted so at the end of 6 or 7 weeks half of the animals were also given 1γ biotin, the other half 1 mg of nicotinic acid daily. It is evident from typical cases in Chart II that biotin provided definite stimulation while the nicotinic acid gave little or no effect.

When vitamin C was omitted from the synthetic diet including 1 γ biotin daily growth was no less than when vitamin C was included, the females averaging 1.40 g per day and the males 1.05 g per day for the 4-week period. Vitamin C was then excluded from the daily vitamin supplement of all our experimental animals on the complete synthetic diet and after 6 months there were no indications of any deficiency.

When the animals were given only 6 crystalline members of the B group (thiamine, riboflavin, nicotinic acid, calcium pantothenate, choline and pyridoxine) plus ½ γ biotin daily, three-fourths of the animals died within 3 weeks and the rest did so poorly that they were discontinued from experiment. In another series when the biotin level was raised to 1 γ per day, the animals also did poorly, one-third to one-half dying within 4 weeks. Since animals did very well on rations containing the 6 members of the B group and 1γ biotin plus inositol and para-aminobenzoic acid it is evident that both or one of these factors are necessary for the nutrition of the hamster.

Table I summarizes the comparative rate of growth of the various synthetic diets and the stock ration.

Discussion. Routh and Houchin[5] reported that the hamster needed nicotinic acid for normal growth. Under our conditions we have obtained good growth when nicotinic acid was excluded from the ration of the hamster. Since Routh and Houchin[5] did not include biotin in their rations it seems probable that they were in reality dealing with a biotin deficiency. Mouth lesions which they reported in riboflavin and thiamine deficient animals were encountered by us in biotin deficient animals which had adequate supplies of these 2 members of the vitamin B group so that probably their experiments were complicated by multiple deficiencies.

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‡ S.M.A. Corp. Conc. No. 200.

[5] Routh, J. J., and Houchin, O. B., *Federation Proc.*, 1942, **1**, 191.

Our data have also shown that vitamin C can be safely excluded from the hamster's ration since no growth differences were obtained when vitamin C was included or omitted from the diet.

Inositol or para-aminobenzoic acid or both may be needed in the ration of the hamster since deaths occurred when both these factors were excluded from the diet. However, experiments are now in progress to determine whether both or only one is needed.

Conclusions. A synthetic ration containing the 6 crystalline members of the B group which will support growth and reproduction in the rat was found wholly inadequate for the hamster. The addition of para-aminobenzoic acid and inositol increased the survival time of the hamster. A definite need for biotin was shown, the animals growing excellently and reproducing when it was included in the ration along with the 8 members of the B group including inositol and para-aminobenzoic acid. On the complete synthetic diet including biotin, inositol, and para-aminobenzoic acid, vitamin C and nicotinic acid are not required by the hamster.

14108

Effect of pH on Stability of Vesicular Stomatitis Virus.

BJÖRN SIGURDSSON.* (Introduced by Carl TenBroeck.)

From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N.J.

Most viruses are rapidly inactivated at high H ion concentrations, and therefore the report of Galloway and Elford[1] that the vesicular stomatitis virus (VSV) was still active after 24 hr at pH 4.51 and 0°C was of interest. Their results were based on qualitative tests, as a convenient method for titrating virus activity was not available at that time. Recently it has been found here that the 7-day-old chick embryo is quite susceptible to VSV inoculated on the chorioallantoic membrane and that it can be used to titrate the virus. Using the embryo to determine the amount of virus, the pH stability of VSV has been determined quantitatively.

The New Jersey strain of VSV originally obtained through the courtesy of Dr. P. K. Olitsky was employed. Seven-day-old chick embryos were inoculated on the membrane with an egg passage of the virus, and the infected embryos were harvested shortly after death. They were ground with 0.9% NaCl solution to make a 10% suspension by weight and the suspension was centrifuged in an angle centrifuge for 10 minutes at 4000 R.P.M. One cc of the supernatant was then added to 9 cc of buffer of the desired pH. The buffer used was the glycine acetate phosphate buffer described by Northrop.[2] It was chosen because it provides a constant ionic concentration in the medium, except for the H^+ and OH^- throughout the pH range studied. This buffer has been used before for a similar purpose.[3] It was found necessary to use the buffer undiluted, as it did not maintain its pH when the tissue-virus suspension was added if it had been diluted 1 part to 5 with saline (tested colorimetrically).

The buffer samples and the virus suspension were mixed in the cold and kept on ice in the refrigerator. Virus activity was then titrated

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* Fellow of The Rockefeller Foundation.

[1] Galloway, I. A., and Elford, W. J., *Brit. J. Exp. Path.*, 1935, **16**, 588.

[2] Northrop, J. H., and DeKruif, P. H., *J. Gen. Physiol.*, 1922, **4**, 639.

[3] Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49.

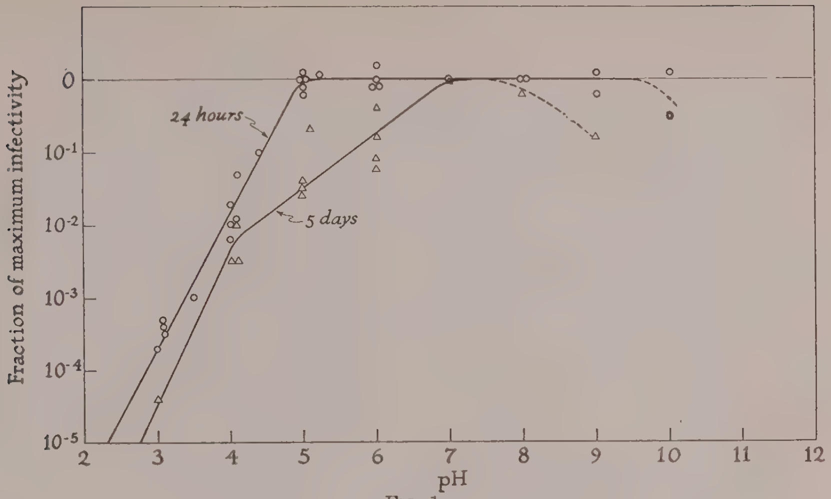

FIG. 1.

24 hours and 5 days later. Control samples with pH 7 were prepared and titrated simultaneously, and the inactivating effect of a given pH was the difference in titer found at the respective pH and pH 7.

For titrating virus activity serial tenfold dilutions of the virus were made in saline buffered to pH 7.4, and five 7-day-old embryos were inoculated on the chorionic membrane with each dilution. The eggs were incubated for 3 days at 35-36°C and the deaths noted. The point of 50% mortality could then be calculated.[4] Very few deaths occurred later than 48 hours after inoculation.

Fig. 1 shows the results of the titrations. After 24 hours below pH 5 the activity decreased rapidly. After 5 days 90% of the virus had been destroyed at pH 6. The irregular form of the 5-day curve is unexplained.

Summary. The activity of VSV suspensions was found unchanged between pH 4 and pH 9 after 24 hours at about 0°C. Increase in pH concentration beyond pH 7 brought about a progressive inactivation of the virus. After 5 days 90% inactivation had taken place at pH 6.

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[4] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

14109 P

Excretion of Exogenous and Endogenous Estrogen in Bile of Dogs and Humans.*

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Data reported previously[1,2] indicate that considerable quantities of exogenous estrogen are excreted in the bile, and the possibility of an enterohepatic circulation of estrogens was suggested. These observations are supplemented by the data presented in this communication.

Human Bile. Bile was obtained by duodenal intubation of 2 women at full-term pregnancy and 3 women 3, 4 and 7 days post-partum, respectively. The following values were obtained by assay[2] of the so-called "C" or liver-bile fraction. Full-term pregnancy: 990 and 600 I.U. free estrogen per 100 cc. Values for free estrogen in blood taken at the same time were 300 and 198 I.U., respectively. Post-partum: 3 days, 450 I.U. (blood 36 I.U.); 4 days, 360 I.U. (blood 18 I.U.); 7 days, 240 I.U. (blood, 9 I.U.).

In a menopausal woman, after withdrawal of control specimens, 1 mg of diethylstilbestrol (120,000 I.U. by the author's assay) was injected intramuscularly and "C" bile was collected in ½-hour fractions for 3½ hours. Controls: inactive; ½ hour, 330 I.U. (total excretion free estrogen); ½-1 hour, 440 I. U.; 1-1½ hours, 650 I.U.; 1½-2 hours, 8200 I.U.; 2-2½ hours, 5360 I.U.; 2½-3 hours, 470 I.U.; 3-3½ hours, 108 I.U. The total excretion in 3½ hours was 15,558 I.U. (13% of activity of administered diethylstilbestrol). Recovery of estrogenic activity in the bile of dogs is considerably lower following administration of diethylstilbestrol than after administration of estrone or estradiol.[2]

Bile Estrogen after Gonadotropins. Two bile-fistula dogs received 4000 units of chorionic gonadotropin (Korotrin)‡ and one 4000 units of pregnant mare serum gonadotropin (Anteron)§ intravenously. Bile was collected for 4 consecutive 24-hour periods. Control specimens were inactive. Total bile estrogen after chorionic gonadotropin: 24 hours, 26 and 80 I.U.; 48 hours, 272 and 105 I.U.; 72 hours, 376 and 132 I.U.; 96 hours, 462 I.U. After pregnant mare serum gonadotropin: 24 hours, 52 I.U.; 48 hours, 246 I.U.; 72 hours, 570 I.U.; 96 hours, 700 I.U.

Absorption of Alpha-Estradiol from Duodenum. 2.5 mg (250,000 I.U.) of alpha-estradiol in sesame oil were introduced into the duodenum (duodenal fistula) in a bile-fistula dog over a period of 8 hours. Bile was collected for four 24-hour periods. The recovery, in terms of percentage of the quantity administered, was approximately as follows: 24 hours: free, 3%; total, 10%. 48 hours: free, 1.3%; total, 10%. 72 hours: free, 0.0%; total, 0.4%. 96 hours: free, 0.0%; total, 0.13%.

Under sodium pentobarbital anesthesia, 3 mg of alpha-estradiol (300,000 I.U.) in 5 cc of bile were introduced into an isolated jejunal loop. After ¾ hour, blood was allowed to flow for ½ hour from the veins draining the loop. The free and total estrogen content of the blood was identical, 6000 I.U. per 100 cc.

Bile Estrogen after Splenic Implantation of Alpha-Estradiol. A 15 mg pellet of alpha-estradiol was implanted in the spleen of a bile-fistula dog. Bile and urine were collected for five 24-hour periods (beginning with the 2nd day) and on the 16th and 23rd days after the implantation. No estrogenic activity was exhibited by the urine at any time during this period. The free estrogen in the bile was as follows: 2nd day, 280 I.U.; 3rd day, 672 I.U.; 4th day, 476 I.U.; 5th day, 550 I.U.; 6th day, 420 I.U.; 16th day, 2025 I.U.; 23rd day, 1780 I.U.

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* Aided by a grant from the Johnson Research Foundation, New Brunswick, N.J.

† J. Ewing Mears Fellow in Medicine and Physiology.

[1] Cantarow, A., Rakoff, A. E., Paschkis, K. E., and Hansen, L. P., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 707.

[2] Cantarow, A., Rakoff, A. E., Paschkis, K. E., Hansen, L. P., and Walkling, A. A., *Endocrinology*, 1942, **31**, 515.

‡ We are indebted to Mr. J. Lucas, of the Winthrop Chemical Company, for the chorionic gonadotropin (Korotrin) used in this study.

§ We are indebted to Drs. Erwin Schwenk and William H. Stoner, of the Schering Corporation, for the pregnant mare serum gonadotropin (Anteron), estrone and estradiol used in this study.

Discussion and Conclusions. Previous observations[1,2] indicate that the bile is an important medium of excretion of exogenous estrogen (10,000-250,000 I.U. doses). The data reported here indicate that large amounts of *endogenous* as well as *exogenous* estrogen are excreted in the bile of human subjects and dogs, and may be present in the bile in comparatively high concentration at a time when the concentration in the peripheral blood and urine is very low. The same is shown to be true following splenic implantation of a pellet of alpha-estradiol. The possibility of the existence of an enterohepatic circulation of estrogen is supported further by the demonstration (1) of the presence of large amounts of estrogen in the mesenteric-vein blood (dog) after instillation of alpha-estradiol into an isolated intestinal loop and (2) of the excretion of estrogen in the bile of dogs following instillation of estrogen into the duodenum.

14110

Biotin Deficiency in Rats Fed Purified Diets Containing Succinylsulfathiazole and p-Aminobenzoic Acid.*

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Only a few reports have been made concerning the effects of succinylsulfathiazole (sulfasuxidine) on the nutrition of animals fed purified diets.[1–5] Welch[1] found no favorable effect on growth of rats when *p*-aminobenzoic acid was added to a purified diet containing sulfasuxidine. In none of the other studies has *p*-aminobenzoic acid (or inositol) been included in the basal diet in attempts to determine the effect of sulfasuxidine on the nutrition of animals.

The effects of sulfanilylguanidine (sulfaguanidine) have been given considerably more attention.[6–11] Through the use of sulfaguanidine or sulfasuxidine deficiencies of biotin,[3,10] folic acid,[2,10] and vitamin K[4] have been reported. The evidence strongly indicates that the vitamin deficiencies which result are due to a lowering by the drugs of microbial synthesis of these essential nutritional factors in the alimentary tract. Black *et al.*[4] found that *p*-aminobenzoic acid counteracts the hypoprothrombinemia and growth inhibition that occurs when sulfaguanidine is fed. Other

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* Supported by a grant from the Graduate Research Fund of Indiana University.

[1] Welch, A. D., *Federation Proc.*, 1942, **1**, 171.

[2] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 713.

[3] Daft, F. S., Ashburn, L. L., and Sebrell, W. H., *Science*, 1942, **96**, 321.

[4] Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P., *J. Biol. Chem.*, 1942, **145**, 137.

[5] Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Pub. Health Rep.*, 1942, **57**, 1559.

[6] Black, S., McKibbin, J. M., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 308.

[7] Mackenzie, J. B., Mackenzie, C. G., and McCollum, E. V., *Science*, 1941, **94**, 518.

[8] Dann, W. J., *J. Biol. Chem.*, 1941, **141**, 803.

[9] Martin, G. J., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 56.

[10] Martin, G. J., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 353.

[11] Light, R. F., Cracas, L. J., Olcott, C. T., and Frey, C. N., *J. Nutrition*, 1942, **24**, 427.

studies[1,4,6,7,11,12] also have indicated that sulfaguanidine is antagonized by *p*-aminobenzoic acid.

In connection with other studies on rats fed both sulfasuxidine and *p*-aminobenzoic acid, we have found evidences of biotin (and a vitamin K[13]) deficiency. This finding constitutes possible additional evidence of a difference between sulfasuxidine and sulfaguanidine as respects their relation to *p*-aminobenzoic acid. Owing to our inability to secure more pure biotin for further study of this problem, we are reporting the results on biotin at this time.

Experimental and Results. Young piebald rats (McCollum strain), 40 to 50 g in weight, were confined to individual cages. The cages were cleaned frequently because the feces of rats fed sulfasuxidine are so soft the screen floors quickly become soiled, thus providing opportunity for coprophagy. The basal diet contained: casein (Smaco, vitamin-free), 18; glucose (Cerelose), 72; salts, 5; corn oil, 3; cod liver oil, 2; and a synthetic vitamin mixture[†] containing thiamine hydrochloride 10 mg; pyridoxine, 10 mg; riboflavin, 20 mg; nicotinic acid, 200 mg; calcium pantothenate, 200 mg; *p*-aminobenzoic acid, 200 mg; inositol, 400 mg; 2-methyl-1,4-naphthoquinone, 600 mg; and choline chloride, 1200 mg. When used, sulfasuxidine[‡] was added at a level of 1% replacing an equal amount of glucose.

Rats fed the basal diet without sulfasuxidine show good growth and appear to be in a moderately good state of nutrition. During the first 6 weeks they gain about 20 g per week. After 10 to 12 weeks they still appear to be almost like stock animals. In contrast, rats fed the basal diet with sulfasuxidine added gain about 10 g per week during the first 5 weeks. From the 5th to 8th weeks about one-half die and the rest eventually succumb. There is a marked variation in ability to survive. Perhaps this is due to differences in ability to utilize the deficient nutritive factors probably being formed in a limited but variable degree by intestinal microörganisms.

The growth inhibition by the sulfasuxidine is considerably reduced by feeding daily 0.5 ml of a liver preparation[§] representing 1.7 g liver.

In Table I it is shown that biotin, either as a concentrate or as the pure methyl ester, effects a significant growth response in rats supplemented daily with 0.5 ml of the liver preparation. In all cases the rats had been restricted to the diet at least 2 weeks before supplementation with the liver. The biotin was not given until liver had been fed 2 weeks. As shown by Daft *et al.*,[3] who used a purified diet deficient in *p*-aminobenzoic acid, certain liver extracts are useful in producing mild degrees of biotin deficiency. In our studies the biotin was administered orally in 1γ daily doses per rat for only 6 or 8 days, after the rats had been on the diet 4 to 13 weeks as indicated. The controls received equal amounts of the biotin solvent, 0.5 ml of 50% ethanol daily.

As shown in Table II, rats fed 1 or 2 γ of a biotin concentrate per day from the time they were restricted to the diet showed a greater growth response when given 0.5 ml of liver preparation daily than did those not given the biotin. Moreover, the total weight of the biotin-fed group was greater than their controls at the time liver supplementation was begun. These results indicate mild biotin deficiency in rats fed both *p*-aminobenzoic acid and sulfasuxidine. But the better growth noted in the biotin-fed animals previous to liver feeding may have been due to "detectable quantities" of folic acid, or other factors in the concentrate, because in another experiment 9 rats showed no appreciable growth response when given pure biotin alone (1 or 2 γ daily for 6 days) after 3 weeks on the basal diet. None of these were given liver.

After 4 to 6 weeks nearly all rats developed

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[12] Wood, W. B., Jr., *J. Exp. Med.*, 1942, **75**, 369.

[13] Wakim, K. G., Day, H. G., Krider, M. M., and O'Banion, E. E., unpublished data.

[†] Most of the synthetic vitamins were supplied through the courtesy of Dr. D. F. Robertson, Merck and Co.

[‡] The sulfasuxidine was kindly furnished by Dr. W. A. Feirer, Sharp and Dohme, Inc.

[§] Prepared from a concentrate generously supplied by the Lederle Laboratories. Four ml of this concentrate is diluted to 40 ml with water to give the preparation as used.

TABLE I.
Effect of Biotin on Growth of Sulfasuxidine-fed Rats after Receiving Liver Concentrate 2 Weeks.

Biotin* supplement	Wks on diet before biotin added	No. of rats, sex	Avg total wt when biotin was added g	Avg gain 1 wk before g	Avg gain 1 wk after g	Avg gain 2 wk after g
5000†	9	2 (0♂)	155	9	15	—
0	9	2 (2♂)	160	8	5	—
5000†	13	3 (0♂)	168	10	23	32
0	13	2 (1♂)	160	10	13	22
Methyl ester	4	3 (2♂)	116	5	24	33
0	4	3 (1♂)	140	7	11	23

* Purchased from S.M.A. Corporation.

† The biotin concentrate contains "detectable quantities of folic acid." (Personal communication from S.M.A. Corporation.)

TABLE II.
Effect of Liver Concentrate on Growth of Sulfasuxidine-fed Rats Receiving Biotin from the Beginning of the Experiment.

Biotin* supplement	Wks on diet before liver added	No. of rats, sex	Avg total wt when liver added g	Avg gain 1 wk before g	Avg gain 1 wk after g	Avg gain 2 wk after g
200†	12	4 (0♂)	145	1	25	41
0	12	2 (0♂)	128	1	15	37
0	9	3 (2♂)	105	3	15	33
0	5	2 (1♂)	91	8	18	35

* Purchased from S.M.A. Corporation.

† The biotin concentrate contains "detectable quantities of folic acid." (Personal communication from S.M.A. Corporation.)

a mild dermatitis characterized by fine, flaky scales on the feet, notably on the anterior surfaces of the hind feet. Alopecia, especially of the abdomen, occurred in over one-half the rats. In a few cases total abdominal denudation occurred. No noticeable improvement in the dermatitis or alopecia followed administration of biotin or liver, but the biotin supplement was given in small doses and for only 6 to 8 days. Daft *et al.*[3] noted remission of dermatitis in rats given biotin for 14 days. "Spectacle eye" was not distinguished with certainty but it appeared to be present in mild form in several animals. There was no definite abnormality in gait as reported by Nielsen and Elvehjem[14] and others. According to these workers a rather severe degree of deficiency, as produced by egg white, must exist before that syndrome appears.

Discussion. Our studies give some support to Welch's[1] conclusion that p-aminobenzoic acid, in the amounts fed, does not abolish the growth inhibiting action of sulfasuxidine. Whether the degree of biotin deficiency in our studies would have been greater with the exclusion of p-aminobenzoic acid from the diet was not determined. However, it is probable that the p-aminobenzoic acid did exert some antagonistic action since we have evidence that it inhibits, but does not totally block, the production of hypoprothrombinemia in rats fed sulfasuxidine.[13] Our animals ingested 1 to 2 mg of p-aminobenzoic acid per rat daily as calculated from food consumption data. Black *et al.*[4] found that 150-300 γ per day was sufficient to counteract the hypoprothrombinemia and growth inhibition produced by sulfaguanidine fed at a 0.5% level.

We believe the relationship of p-aminobenzoic acid to the action of sulfasuxidine and sulfaguanidine in the alimentary tract needs to be investigated on a quantitative basis. As shown by Wood,[12] working with 6 different sulfonamides and pure cultures of *B. coli*, the bacteriostatic potency of a sulfonamide is directly proportional to its ability to counteract the antibacteriostatic action of *p*-aminobenzoic acid. If, therefore, sulfasuxidine should happen to be appreciably more bacteriostatic than sulfaguanidine a greater amount of *p*-aminobenzoic acid might be required to exert a comparable antibacteriostatic action when the former is fed. Since Martin[10] has found that biotin improves the growth of sulfaguanidine-fed rats receiving *p*-aminobenzoic acid it must be concluded that, at the levels fed, the antagonistic action of the latter is not complete in the case of sulfaguanidine. Similarly our studies indicate that sulfasuxidine lacks considerable of being totally inhibited by *p*-aminobenzoic acid even at the seemingly large levels we have fed.

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[14] Nielsen, E., and Elvehjem., C. A., *J. Biol. Chem.*, 1942, **144**, 405.

Summary. Biotin deficiency occurs in rats fed a purified diet containing sulfasuxidine and *p*-aminobenzoic acid. Growth appears to be a sensitive indicator of biotin deficiency in the rat.

14111

Sugar Alcohols. XXIII. Effect of Sorbitol Feeding on Successive Generations of Rats.

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In former studies[1] two of the authors showed mannitol and sorbitol to be innocuous when fed in comparatively large amounts to the *Rhesus macacus* monkey and man over long-time periods. The laxative action of large quantities of mannitol limits its value as a dietary adjunct. Sorbitol, however, does not possess this disadvantage and at present is enjoying a widespread use as a carbohydrate-like item of diet. It seemed fitting, therefore, to study the effect of sorbitol feeding on successive generations of animals.

Material and Methods. Crystalline sorbitol was used in one group of studies. In the other, a commercial sorbitol syrup was employed. This syrup is a non-crystallizing aqueous solution of sorbitol having a polyhydroxylic compound content of about 83%. It is manufactured by the electrolytic reduction of glucose and is known under the trade name of "Arlex."* Each of these substances mixed with Purina chow meal was fed to young white rats of our laboratory stock and also animals of the Duke University strain. Controls were fed dextrose and the basal diet. Each compound composed 5% of the total food intake. Males and females were kept separated until they were at least 100 days old and then mated. This was repeated through 3 generations. Growth curves of the rats fed the 3 types of food in the 3 generations were plotted. Periodically animals were killed for gross and histologic study. At the end of 1 year the capacity of the livers to store glycogen was determined. Eighty, first generation animals fed the experimental diet from the time of weaning were used and the experiment was extended over 20 months.

Results. The growth curves of the first generation animals fed the 3 kinds of food were identical within the normal variations of the experiment. The offspring of these animals showed similar growth rates in the second and third generations. The litter sizes were about the same. The average liver-glycogen[2] content of the first generation animals after 1 year was: dextrose-fed 1.9% (6 animals), sorbitol-fed 3.5% (7 animals), and arlex-fed 3.8% (3 animals). Animals from this group previously fasted for 48 hours showed the following values for liver glycogen: dextrose-fed 0.33% (10 animals), sorbitol-fed 0.44% (6 animals), and arlex-fed 0.22% (4 animals). Grossly and histologically the kidney, liver, spleen, pancreas, and duodenum of these animals in the 3 generations showed no abnormalities deemed attributable to the dietary regimen.

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[1] Ellis, F. W., and Krantz, J. C., Jr., *J. Biol. Chem.*, 1941, **141**, 147.

* Generous supplies of crystalline sorbitol and "Arlex" were furnished by the Atlas Powder Co. of Wilmington, Del.

[2] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

Summary. Comprising 5% of the food intake, neither sorbitol nor arlex affects deleteriously the rate of growth, liver-glycogen storing capacity or important metabolic viscera of the white rat through 3 generations.

14112 P

Size of Infective Particle and Hemagglutinin of Influenza Virus as Determined by Centrifugal Analysis.

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It has recently been shown[1] that sedimentation boundaries could be obtained and approximate size determinations made with dilute protein suspensions in the angle centrifuge if a synthetic density gradient of sucrose or some other nonsedimentable material was added to counteract disturbances of convection. Under these conditions tubes could be removed from a centrifuge and sampled without greatly altering a boundary. These principles have been applied to the sedimentation of influenza virus in allantoic fluid, and the findings are herewith reported.

The PR8 strain of influenza A virus or the Lee strain of influenza B virus was inoculated into the allantoic sacs of 11-day-old White Leghorn embryos. After 48 hours' incubation at 37°C the eggs were chilled overnight at 4°C; the blood-free allantoic fluids were removed and cleared by centrifugation at 1800 r.p.m. for 5 minutes. Without further treatment the virus fluids were spun in celluloid tubes in a vacuum air-driven centrifuge in the presence of a sucrose density gradient with the concentration of sugar increasing from a negligible amount at the top of the fluid column to nearly 12% at the bottom of the tube. After centrifugation at a given rate 1 cc samples were removed at different levels in the fluid column by means of a sampling apparatus already described.[2] Control tubes which had been prepared in identical manner but not centrifuged were allowed to stand for a comparable time and also sampled. All the samples were immediately tested for infectivity in mice and for capacity to agglutinate chicken red blood cells.[3]

The results of a typical experiment are shown in Fig. 1. Repeated tests with allantoic fluids containing either A or B virus strains after centrifugation at 12,800 r.p.m. for 28 minutes showed the following characteristics: (1) a marked drop in virus titer in the upper cc of the fluid column to about 0.5% of the original concentration, (2) a sharp rise in virus concentration (a boundary indicating sedimentation of a large group of virus particles of about the same size) slightly more than one-third of the way down the tube followed by (3) a region of uniform or very gradually increasing concentration, and (4) a marked increase in concentration in the bottom sample due to deposition of the virus. The displacements of the boundary from the meniscus were roughly proportional to the amount of centrifugation. For example, the sedimenting virus boundary moved only one-sixth of the tube length when the centrifugation time was decreased to 14 minutes, whereas when increased to 56 minutes at the same speed it moved almost to the bottom of the tube. In the latter instance careful sampling revealed that less than 0.1% of the original concentration of virus remained in the upper cc of the fluid column as measured by infectivity tests.

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[1] Pickels, E. G., *J. Gen. Physiol.*, 1943, **26**, 341.

[2] Hughes, T. P., Pickels, E. G., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1938, **67**, 941.

[3] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

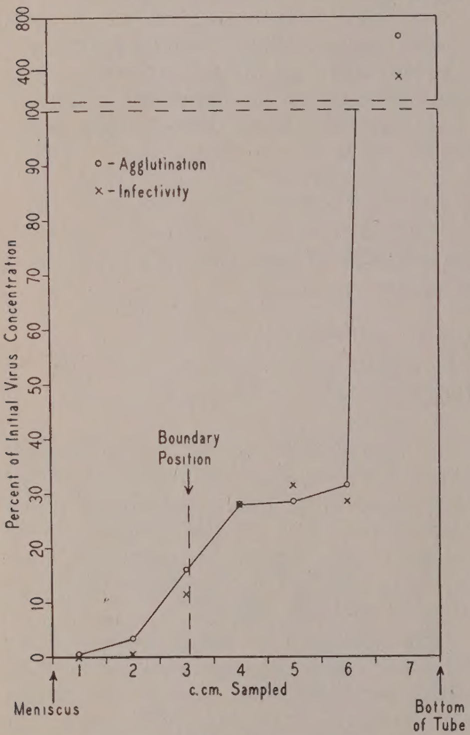

FIG. 1.

Sedimentation of infective particle and hemagglutinin of influenza B virus in allantoic fluid. Samples were taken from celluloid tubes (diameter = 1.3 cm) inclined at 35° to axis of rotation, after 28 minutes at 12,800 r.p.m. Meniscus of fluid was 6 cm from axis of rotation.

Although the sedimentation rate of boundaries obtained with allantoic fluids containing either A or B virus strains was approximately the same, differences in the character of the sedimentation curves were observed which suggest varying amounts of aggregation. The sucrose solutions had no detectable effect on the virus controls. Comparable tests in which allantoic fluids were centrifuged without a density gradient showed more virus (several per cent of the original concentration) in the upper part of the tube and a gradual increase in virus concentration down the tube without exhibiting a sedimenting boundary.

Determinations of the approximate particle size of influenza virus were made from measured displacements of the boundary from the meniscus after several different centrifugation times and by application of an equation already derived for the angle centrifuge.[1] Assuming the usual protein density value of 1.33, the length (or diameter, if spherical) of the influenza virus particle in allantoic fluid was found to be at least 60 mμ. Even if an abnormally high density value is assumed, the size determination is altered by only a few percent. Confirmation of the sedimentation rate measured in the angle centrifuge was obtained by absorption (Fig. 2) and refractive index photographs taken with concentrated and purified virus preparations in the ultracentrifuge. These results indicate that the actual size of influenza virus in allantoic fluid is nearer the value reported in the original filtration experiments with mouse lung extracts (80-120 mμ)[4] than that recently ascribed to the virus in allantoic fluid (10-15 mμ).[5] The physical properties of the virus will be more fully described in later reports.

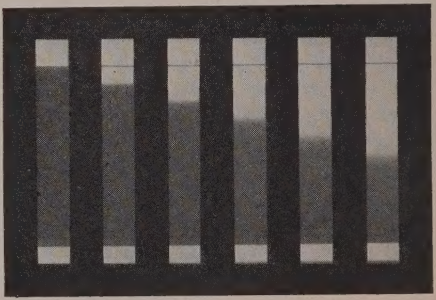

FIG. 2.

Absorption photographs showing sedimenting boundary of influenza B virus obtained from allantoic fluid. Speed 6300 r.p.m.; interval between photographs 10 minutes. Virus purified and concentrated by adsorption and elution from chicken red blood cells and subsequent centrifugation.

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[4] Elford, W. J., Andrewes, C. H., and Tang, F. F., *Brit. J. Exp. Path.*, 1936, **17**, 51.

[5] Chambers, L. A., and Henle, W., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 481.